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Conjugal Transfer of the Catabolic Plasmids TOL and NAH7.

by

Poovadan Chikoli Anoop ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"Conjugal Transfer of the Catabolic Plasmids TOL and NAH7"** submitted by **Poovadan Chikoli Anoop** in partial fulfillment of the requirements for the degree of **Master of Science** in **Microbiology and Biotechnology.**

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Abstract

The spread of plasmid borne pathways involved in the degradation of organic pollutants suggests that plasmids are important tools in the dissemination of degradative genes. Conjugation is probably the most important mechanism of gene transfer in the soil. This study focussed on the horizontal transfer of the plasmids TOL and NAH7, encoding degradation of toluene and naphthalene respectively, on agar surfaces and under different physiochemical conditions in sand microcosms. Spontaneous deletions and insertions occurred in TOL and NAH7 plasmids respectively as a result of horizontal transfer, but these events did not affect the degradative phenotype associated with the plasmids. Higher initial parental densities and a 1:5 and 1:10 donor:recipients positively influenced conjugation frequencies. Studies on conjugal transfer revealed that the initial increase in transconjugants resulted possibly from horizontal transfer and subsequent increases could be due to vertical transfer as well as horizontal transfer. Nutritional selection and pH range of 6.6-7.2 resulted in higher conjugation frequency. Optimum transfer of NAH7 and TOL were detected at 25° and 30°C respectively. Green fluorescent protein (GFP) was used as a biomarker to detect horizontal transfer of these plasmids. Attempts were made to isolate catabolic plasmids by endogenous and exogenous isolation from hydrocarbon-contaminated sites but the degradative functions in the degraders apparently were not plasmid-borne.

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Dedicated to my Supervisor and

all my teachers

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List of Abbreviations

ATCC	American Type Culture Collection
bp	base pair
C	carbon
cfu	colony forming units
ddH_2O	doubly distilled water
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
ds	double stranded
EDTA	ethylene diamine tetra acetic acid
$FeSO_4 \bullet 7H_2O$	ferrous sulphate heptahydrate
GFP	green fluorescent protein
kbp	kilobase pair (s)
NaOAc	sodium acetate
NH_4OAc	ammonium acetate
PAH	polycyclic aromatic hydrocarbon
SDS	sodium dodecyl sulphate
ss	single stranded
SSPE	sodium chloride in sodium phosphate/EDTA
TAE	Tris/acetate/EDTA
TBE	Tris/borate/EDTA
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

Antibiotics

Ap	ampicillin
Cm	chloramphenicol
Km	kanamycin
Nal	nalidixic acid
Rif	rifampicin
Sm	streptomycin
Sp	spectinomycin
Tc	tetracycline

Media

BH	Bushnell-Haas
LB	Luria-Bertani
MM	mineral medium
PCA	Plate count agar

Chapter 1

Introduction and Literature Review

1.1. General Introduction

The widely perceived classical route of gene transfer in bacteria is the passage of bacterial DNA from mother to daughter cell, a process known as vertical gene transfer. However, bacterial cells can also transfer genetic material by a process known as horizontal gene transfer wherein genetic information is passed from one microorganism to another. Extrachromosomal genetic elements such as plasmids, transposons and bacteriophages play a major role in horizontal transfer of genetic material.

The widespread use of antibiotics, and increased industrial and agricultural activities resulting in contamination of the environment have posed evolutionary challenges for bacteria. Gene loss and reacquisition may be important in evolution (Lawrence and Roth, 1996). Genes transferred horizontally between bacteria, like other sources of genetic variation, are subsequently subject to natural selection. During bacterial evolution, the ability of bacteria to exploit new environments and to respond to new selective pressures can often be more readily explained by the acquisition of new genes by horizontal transfer, rather than by the sequential modification of gene function by the accumulation of point mutations (Syvanen, 1994; Davison, 1999). The rapid evolutionary changes possible in a bacterial population through gene transfer transcend the changes envisioned by traditional Darwinian selection (Tauxe et al., 1989).

The study of microbial gene transfer, including how bacteria acquire and disseminate genes, will increase our understanding of the role of horizontal transfer in bacterial evolution, ecology and also for risk assessment due to environmental releases of plasmid-encoded genes. From a fundamental point of view, there is a need to explore how DNA uptake influences the adaptation of microorganisms to toxic substances and changing environmental conditions. The increasing interest in the introduction of genetically engineered microorganisms into the environment has also contributed to the number of studies on horizontal gene transfer.

The introduction of microorganisms into natural environments for bioremediation is a topic which has evoked a lot of interest. Bioremediation is an ecologically sound natural process and the residues are usually harmless products. Bioremediation is less expensive than physicochemical methods and can be used along with a wide range of physical and chemical technologies to increase the efficacy of the process. There are two basic types of natural bioremediation: (1) Biostimulation, which provides nutrients and suitable physiological conditions for the growth of the indigenous microbial populations. This in turn results in degradation of contaminants owing to increased metabolic activity; and (2) Bioaugmentation, which introduces laboratory grown microorganisms into a polluted environment. Horizontal transfer of catabolic plasmids among stimulated natural microflora or from introduced bioremediative bacteria to the indigenous microflora could be advantageous in bioremediation. Horizontal transfer of antibiotic resistance plasmids has been well studied but studies of the transfer of degradative plasmids are scarce.

1.2. Horizontal gene transfer

Gene transfer among different genera and species of bacteria has long intrigued environmental microbiologists. The significance of this promiscuous horizontal gene transfer is that it provides a huge pool of genes for bacterial evolution. Horizontal transfer may in fact be instrumental in bacterial evolution since it allows an organism to acquire a well-adapted and developed capability. Such transfers have probably contributed to the evolution of bacteria, since phylogenetically distant microorganisms have been demonstrated to exchange genetic material (Trieu-Cuot et al., 1987). The most well-documented examples of horizontal gene transfer are found in the field of clinical microbiology, namely the transfer of antibiotic resistance plasmids. Early evidence for the horizontal exchange of antibiotic resistance genes came from studies that monitored the distribution of such plasmids isolated from organisms present in geographically well-separated areas. Nowadays, owing to the interest in the fields of bioremediation and introduction of genetically engineered microorganisms, some attention is focussed on the aspect of horizontal transfer of degradative plasmids.

A number of host-associated factors affect horizontal gene transfer (Saunders et al., 1990). Restriction of foreign DNA provides a significant barrier to gene transfer, and DNA not specifically modified by methylation is generally cleaved on entry into a restrictive host. Another barrier to gene exchange is damage to incoming DNA or prevention of DNA entry by surface exclusion receptors. The ultimate host range of a plasmid might also be limited by a requirement for a specific biochemical infrastructure or plasmid incompatibility within the recipient. Some bacteria also lack the correct replicative machinery for the maintenance of plasmids after transfer. Even after successful plasmid transfer, the DNA might be subject to recombination events in the host cell which might lead to destruction or integration into the chromosome of the host bacteria.

Mechanisms that enable horizontal transfer between individual cells appear to be a ubiquitous feature of bacteria. There are three main types of DNA transfer: transformation, transduction and conjugation. These mechanisms provide the basis for gene transfer in the environment and combinations of these mechanisms might bring about horizontal gene transfer.

1.2.1. Transformation

The oldest studied mechanism of introducing DNA into microorganisms is transformation (Griffith, 1928), which involves the uptake of any naked DNA by competent bacterial cells. Transformation is a normal physiological function of certain bacteria and is mediated by chromosmal genes (Smith et al., 1981). Transformation has been shown to occur in several environments such as soils and biofilms (Graham and Istock, 1978; Lorenz and Wackernagel, 1991). Therefore, any DNA released in soil from microorganisms theoretically can be taken up by other microorganisms. Competence is a specific physical state of bacterial cells that allows nucleic acids to bind to the cell surface and be internalized by the host (Mazodier and Davies, 1991). Competence seems to be internally regulated, being greatest in exponential phaseand least in stationary phase (Paul, 1999). Some bacterial species can be efficiently transformed by bacterial DNA of any origin while others are more selective, specifically taking up DNA from their own species. The release of DNA from microorganisms usually occurs only after cell lysis Despite its sensitivity to nucleases, DNA is relatively common in almost all environments and such DNA adsorbed to sand and clay particles retains its transforming ability for weeks or even months (Chamier et al., 1993; Lorenz and Wackernagel, 1994; Davison, 1999).

Plasmid transfer in soil can include conjugation and transformation. In transformation experiments, the concentration and size of transforming DNA is an important factor (Trevors and van Elsas, 1992). Natural transformation in soils involves the release of DNA, persistence and adsorption of DNA to soil particles, protection of adsorbed DNA against exonuclease activity, presence of competent bacteria and transformation by DNA adsorbed to soil particulates. Soil has the potential to promote the horizontal transfer of extracellular DNA to bacteria via transformation but transformation frequencies are low, making detection difficult in nature or in microcosms (Paget and Simonet, 1994). Rochelle et al. (1988) showed that 7.8 kb plasmids encoding resistance to mercury isolated from natural *Acinetobacter* isolates were capable of transforming other *Acinetobacter*. Paget and Simonet (1996) confirmed that extracellular DNA adsorbed on a soil matrix composed of minerals and organic matter could still transform bacteria under environmental conditions. In soil, *Acinetobacter calcoaceticus* could only be transformed in nonsterile microcosms when excess nutrients were present (Nielsen et al., 1997).

Williams et al. (1996) demonstrated natural transformation in open experiments incubated in river epilithon and found that transfer frequency generally increased with temperature. Frischer et al. (1994) showed natural transformation of indigenous marine bacteria by using plasmid multimers of pQSR50, an antibiotic resistance plasmid. Paul et al. (1991) detected transformation by a broad-host-range plasmid in a marine *Vibrio* sp. in unamended non-sterile marine water columns. Nutrients facilitated transfer probably by stimulating recipient growth and by providing a preferred carbon source; at low nutrient conditions, the DNA was used as a nutrient source and was not available to transform the recipients (Paul et al., 1991; Paul, 1999). Paul et al. (1992) also reported intergeneric natural plasmid transformation between *E. coli* and a marine *Pseudomonas* sp.

Naturally occurring transformation in bacteria has been observed in several genera and appears to provide only limited opportunities for genetic exchange (Stewart and Carlson, 1986). Though gene transfer frequencies by transformation is very low, this mechanism can also account for alteration of DNA and contribute to the evolutionary process.

1.2.2. Transduction

A wide range of bacteriophages has been identified in many bacterial genera. Transduction involves attachment of a bacteriophage containing bacterial genes to a specific surface on the bacterial surface, injection of its DNA into the host, and integration of the DNA in the bacterial chromosome (or autonomous replication when a replicative plasmid is transduced). Packaging of host genomic DNA in phage heads occurs with either random fragments or specific segments linked to phage DNA that subsequently may be transferred to new recipients. These are known as generalized and specific transduction and can be mediated by double stranded (ds) DNA phages. In generalized transduction, any bacterial gene might be transferred whereas in specialized transduction, genes located near the site of prophage integration are transferred preferentially.

Jiang and Paul (1998) stated that phages are common in the environment and are stable because they are protected by their protein coats. They described plasmid transduction of a lysogenic marine phage host system at very low transfer frequencies (10^{-9} to 10^{-7}). Another phage (UT1) isolated from a fresh water habitat was able to transduce *P. aeruginosa* and also members of the indigenous populations of natural lake-water environments (Ripp et al., 1994). Phage P1 of *Escherichia coli* has inter-genus DNA transfer capability and can also introduce DNA into *Myxococcus* spp. (Kaiser and Dworkin, 1975; Murooka and Harada, 1979). However, transduction between unrelated strains may be limited by restriction endonucleases, which recognize specific DNA sequences and, if these are not modified by methylation, cleave the DNA. Moreover there is a high specificity of interaction between most bacteriophages and their bacterial receptor sites (Reanney et al., 1983). Also, transducing phage released from the host cells usually contain a mixture of phage genomes with some host DNA. If the phage is virulent, newly infected host cells may lyse before any transduced DNA can integrate. Most of the evidence presented on transduction indicates that this could be an important mechanism of gene transfer in marine ecosystems whereas transduction in soils might occur at too low frequencies to be important.

1.2.3. Conjugation

Conjugation is the primary route of broad host range DNA transfer between different genera of bacteria, and many plasmids are transferred by this mechanism. Plasmids are small circular double stranded DNA molecules capable of autonomous replication, present as a few copies (1-4, low copy number) or many copies (10-40, high copy number) in one cell, depending on the size of the plasmid i.e., small plasmids are present in higher copy numbers and vice versa. The life cycle of plasmids comprises replication and distribution of the plasmid copies to the daughter cell. A third event i.e., horizontal transfer, can also be included.

Conjugation is a very versatile process that allows different types of bacteria to mate. Paul (1999) stated that conjugation is viewed as the most promiscuous of the different gene transfer mechanisms, showing the least restriction in relatedness of host and recipients. In conjugation, plasmids as well as parts of the chromosome can be transferred. Plasmids capable of mediating their own transfer and of mobilizing other replicons have been found throughout the Eubacteria and exhibit great diversity. Although there are other self-transmissible conjugative elements called transposons, plasmids are the most studied conjugative elements.

There are numerous examples of inter-genus and even inter-kingdom DNA transfer via conjugation (Trieu-Cuot et al., 1988; Heinemann and Sprague, 1989; Winans, 1992; Bundock and Hooykaas, 1996; Gebhard and Smalla, 1998), however gene transfer between different bacterial genera is possible but is likely to occur at low frequency rates (Frost, 1992). A barrier for natural horizontal gene transfer is the cell wall type. Transfer between Gram-positives and Gram-negatives is limited, possibly due to the different cell walls, which prevents genetic exchange under natural conditions (Tschape, 1994). The rates of plasmid transfer differ based on the type of plasmid and the host bacterium.

Since bacteria have been found to lack sexual reproduction and meiotic exchange, plasmids are today understood as an essential evolutionary compensation for recombination and genetic acquisition of new properties and capabilities (Felsenstein, 1974; Reanney, 1976; Eberhardt, 1989). Plasmids themselves are symbionts and serve only accidentally as carriers or vehicles for horizontal gene transfer, at least when favored through specific selection pressure. Plasmid functions necessary for horizontal transfer are the capability for self-transmission and for integrating foreign DNA. These mechanisms allow plasmids to endow their host bacteria with genetic variability and adaptation to environmental stresses (Tschape, 1994). Conjugation-mediated recombination between strains must be a major factor in the evolution of bacterial species. For example, plasmids encode resistance to antibiotics, heavy metals, catabolism of natural organic compounds and man-made xenobiotics.

Plasmids are divided into incompatibility groups based on sharing of either replication control or partitioning functions. Two plasmids that are very similar and cannot be maintained together in the same cell are termed incompatible and are classified into the same incompatibility group. Compatible plasmids can co-exist in the same cell as independent replicons for many generations without either of them being lost. Incompatibility groups IncP, IncC, IncQ, IncN, IncW, IncJ, IncM have been classified using enteric bacteria as hosts. The incompatibility groups termed IncP1, IncP2,-IncP16 have been determined using the fluorescent pseudomonads as hosts (Jacoby and Shapiro, 1977).

Conjugative plasmids vary in size from molecules of several hundred kilobases carrying genes for many complex functions (e.g., symbiotic plasmids in *Rhizobium*) to the other smaller natural plasmids (<10 kb) encoding limited functions (e.g., 9 kb *Streptomyces* plasmid pIJ101). The typical conjugation event involves transfer of plasmid DNA from a cell harboring a plasmid, called the donor, to a cell which does not have the plasmid, termed the recipient. Plasmid transfer frequencies are estimated as a ratio of transconjugant to donor or recipients. Conjugation involves cell-cell recognition and pairing often made by sex pili, formation of a pore for DNA to pass through, nicking one strand of the plasmid DNA in the donor and synthesis of a new plasmid copy to transfer as single stranded (ss) DNA to the recipient where it is converted to a ds circle (Hirsch, 1990). Any plasmids having only a limited host range are called narrow host range plasmids (e.g., plasmids that can only replicate in *E.coli* and related enteric species, or only in *Mycobacterium* spp.). Some plasmids (including F) appear to have a limited host range. Plasmids able to replicate in a number of genera are designated broad host range plasmids. Broad host range plasmids carry more of the genes required for their replication and therefore are less dependent on specific host replication proteins. IncP, IncQ and IncW plasmids are considered to have a very broad host range.

The IncP plasmid conjugation systems are widely used as tools for gene transfer and the conjugative machinery serves as a model for studying the mechanism of broad host range plasmid transfer. The IncP conjugation system possesses specific properties of broad-host-range transfer that is not shared by the F-plasmid. Detailed analysis of the RK2/RP4 conjugation system revealed that the IncP transfer system conforms to the basic model of conjugation proposed for F- and related plasmids. Transfer proceeds in a polarized manner and *in vitro* nicking at *oriT* (origin of transfer) by the RK2/RP4 relaxosome is believed to be the correlate of the *in vivo* single strand nick that initiates the transfer process. Interactions between the *oriT* sequence and the conjugation system are highly specific and require at least three plasmid-encoded gene products, including an 11 kDa protein that binds specifically to *oriT*. The IncP plasmid DNA primases are products of the TraI region and function in DNA transfer, and transfer of the primase to the recipient during conjugation might function in priming of the DNA strand complementary to the transferred strand. Both DNA strands from the *oriT* region can serve as initiation sites for complementary strand synthesis mediated by the RK2/RP4 primase, implying that these enzymes could prime conjugative DNA synthesis in both the donor and the recipient. The control of conjugal transfer gene expression in IncP plasmids remains unclear and the high transfer frequencies of native IncP plasmids indicate that the transfer phenotype is constitutive (Barth et al., 1978; Guiney and Lanka, 1989). The broad-host-range capability of the P- plasmids reflect specific adaptations at the level of both mating pair formation (Mpf) and DNA transfer and replication (Dtr), and P-pili are likely to be crucial for broad-host-range transfer (Guiney, 1982; Guiney and Lanka, 1989).

Plasmid transfer and the associated mobilization of chromosomal DNA or other replicons may be immune to host restriction endonucleases because the DNA enters the cell in ss form, but there are other constraints. Donor and recipients must make contact and form a pair, which involves specific recognition. Another barrier can be the presence in the recipient of a similar plasmid which gives rise to surface exclusion and incompatibility (Hirsch, 1990).

Most plasmids are capable of self-transfer (Tra$^+$) and mediate their own transfer because they carry the *oriT* site, where transfer replication is initiated, and *tra* genes required for mating pair formation, initiation, stabilization and continuation of transfer replication. The non self-transmissible plasmids (Tra$^-$) can be mobilized *in trans* by the transfer functions encoded on a suitable conjugative plasmid. At present, there are three different models of plasmid mobilization:

1. Direct mobilization: The Tra$^+$ and Tra$^-$ plasmids reside in one and the same donor cell, which mates with a plasmid free recipient. The Tra$^-$ plasmid is transferred by the transfer factors of the Tra$^+$ plasmid provided *in trans.*
2. Triparental mobilization: This describes a three way mating involving a donor of the Tra$^-$ vector, a helper cell harboring a Tra$^+$ plasmid, and a plasmid-free recipient (Stotzky and Babich, 1986). Triparental exogenous isolation is a method that enables the selection of plasmids based on conjugative properties as a phenotype (Top et al., 1994).
3. Retrotransfer, or retromobilization, or gene capture: Retrotransfer is the ability of some conjugative plasmids to mobilize chromosomal markers or a non-conjugative plasmid not only in the classical forward direction, i.e., from donor to recipient of the conjugative plasmid, but also in the opposite direction, i.e., from recipient to donor of the conjugative plasmid (Mergeay et al., 1987). This ability is reported mostly with plasmids belonging to the IncP group. Heinemann et al. (1996) proposed that retrotransfer of genes to donors during conjugation remained genetically and physically indistinguishable from two successive rounds of conjugation between neighbors. Hill et al. (1995) reported a high incidence of retrotransfer amongst plasmids isolated from epilithic bacteria and some of these plasmids retrotransferred an IncQ plasmid at very high frequencies.

Cointegration occurs when two different circular plasmids fuse to become one. Thereby, a non-self-transmissible nonmobilizable plasmid may be sexually transferred due to the action of its cointegrated self-transmissible partner. Such plasmid fusion is often facilitated by the presence, on one of the plasmids, of insertion elements or transposons, and resolution of the cointegrate occurs in the recipient cell. Conjugation may also be effected by conjugative transposons which may facilitate plasmid mobilization and cointegrate formation (Clewell et al., 1995; Davison, 1999).

1.3. Presence of plasmids in polluted environments

Increased industrial, agricultural and human activities have resulted in an increase in polluted environments. Specific adaptations to pollutants are often plasmid-borne. Like multiple drug-resistance markers both in enteric and other pathogenic bacteria, the metabolic versatility of soil bacteria, with few exceptions, is determined totally or partly by catabolic plasmids (Chakrabarty, 1976). The term metabolic or catabolic or degradative plasmid refers to plasmids carrying genes encoding enzymes that catalyze multistep reaction sequences leading from noncellular structures to anaplerotic intermediates. It also denotes the regulation of these predominantly energy-coupled processes, yielding principally the 2 to 4 carbon acids whose metabolism is chromosomally encoded. Most known metabolic plasmids are self-transmissible and range in size from ca. 100 to >200 kb (Gunsalus, 1985). The spread of plasmid encoded pathways involved in the degradation of organic pollutants suggests that plasmids are important tools in the dissemination of genes and in the evolution of new functions under selective pressure (Harayama et al., 1987; Burlage et al., 1990; Sayler et al., 1990). Conjugation of large catabolic plasmids under environmental conditions may be less frequent but nonetheless effective (Ramos-Gonzalez et al., 1991; Top et al., 1995).

Hada and Sizemore (1981) isolated 473 *Vibrio* spp. from an underwater oil field in the Gulf of Mexico and compared the frequency of plasmid occurrence with strains isolated from an unpolluted control area 8 km distant. They found that 35% of the isolates from the oil field contained plasmids compared to 23% in the control area.

Mergeay et al. (1985) isolated *Alcaligenes eutrophus* CH34, the first gram-negative bacterium with plasmid encoded resistance to heavy metals, from a decantation tank of a zinc factory in Belgium. Hardman et al. (1986) found large plasmids, encoding catabolism of halogenated carbon acids and resistance to one or several of the heavy metals (mercury, selenium and tellurium) in organisms isolated from polluted sites. Timoney et al. (1978) examined the heavy-metal resistance patterns of *Bacillus* isolates and suggested that heavy-metal resistance was plasmid mediated.

The study of bioremediation techniques involves the isolation and characterization of naturally occurring microorganisms with bioremediation potential. The next step is to study the catabolic activity and genetically characterize the organisms. Endogenous plasmid isolation is the most commonly adopted method for plasmid isolation. This is a method that first screens for strains that have a specific phenotype, known to be often plasmid-borne, and then analyzes the isolated strains for their plasmid content by plasmid extractions and/or by transfer to other bacteria. The advantage of this method is that the host and the plasmids are isolated together and thus the plasmids should have direct relevance to the organisms they were isolated from. The disadvantage of this method is that nothing is known about the relevance of the plasmids to gene exchange in the ecosystem being studied (Fry and Day, 1990). Therefore another method, called exogenous plasmid isolation, has been developed by Bale et al. (1987,

1988) who successfully isolated mercury resistance plasmids from the epilithon of the River Taff. Hill et al. (1992) also isolated many mobilizing plasmids from the epilithon by this procedure. This method involves mixing a suitable plasmid-free recipient strain with a natural bacterial community, incubating this mixture to allow mating to occur, and selecting for recipient cells that have gained a new marker (Fry and Day, 1990). Top et al. (1994) assessed the plasmid mobilization potential of a soil ecosystem on the basis of the number of transconjugants obtained after exogenous isolation. They indicated that broad host range mobilizing plasmids belonging to incompatibility groups other than IncP might be present in polluted Essen soil. Top et al. (1993) demonstrated that, despite the presence in polluted soils of degradative strains harboring catabolic plasmids, these plasmids could not readily be isolated by exogenous isolation owing to a narrow transfer, replication or expression range.

1.4. TOL plasmid

Pseudomonas strains often carry plasmids regulated by natural and synthetic organic residues that bear primary roles in their mineralization (Gunsalus and Yen, 1981). Bacterial genes encoding degradation of lower molecular weight hydrocarbons, such as benzene, toluene, naphthalene and biphenyl commonly are borne on plasmids such as those belonging to the TOL and NAH families (Burlage et al., 1989; Yen and Serdar, 1988).

The best understood catabolic plasmid is the TOL plasmid which was described originally in *Pseudomonas arvilla* mt-2, a strain now referred to as *P. putida* mt-2 (Williams and Murray, 1974; Burlage et al., 1989). The archetypal TOL plasmid pWW0

(117 kb), is self-transmissible and belongs to the IncP9 group. Catechol could be oxidized either through intradiol fission by catechol 1,2-oxygenase (termed *ortho* cleavage) or through extradiol fission by catechol 2,3-oxygenase (*meta* cleavage) (Dagley et al., 1960). TOL encodes 12 genes responsible for the degradation of toluene and the *m*-and *p*- xylenes by a pathway that involves *meta* cleavage of the aromatic ring (Worsey and Williams, 1975) while the chromosomal genes encode the *ortho* pathway (Figure 1.1). The enzymes and chemical intermediates of toluene degradation by TOL plasmid are shown in Figure 1.2 and Table 1.1. The catabolic genes of pWW0 encoding the 12 enzymes are organized within two regulons, regulated by *xylS* and *xylR* regulators, referred to as the upper and lower pathway, contained within a 40 kb segment (Worsey et al., 1978; Nakazawa et al., 1980; Inouye et al., 1981; Franklin et al., 1981). The upper pathway, *xylCAB,* encodes the degradation of toluene and xylene to benzoate and toluate (Harayama et al., 1986) and the lower pathway, *xylDLEGFJKIH*, encodes the degradation of benzoate and toluate to acetaldehyde and pyruvate (Harayama et al., 1984). Substrates for the upper-pathway enzymes, such as toluene or *m*-methyl benzyl alcohol, are activators of the pathway when *xylR* is present, whereas *m*-toluate is both a substrate and an inducer for the lower pathway in conjunction with the *xylS* gene product. However, upper-pathway substrates can activate the lower pathway if both *xylR* and *xylS* are present (Figure 1.3; Burlage et al., 1989). Cuskey and Sprenkle (1988) demonstrated that the lower pathway could be induced by benzoate alone, without either regulatory protein, suggesting the involvement of a chromosomal regulator.

Cells lacking the *meta* pathway can be isolated after growth on benzoate, which is an intermediate of the *meta* pathway and also of the chromosomally-encoded *ortho*

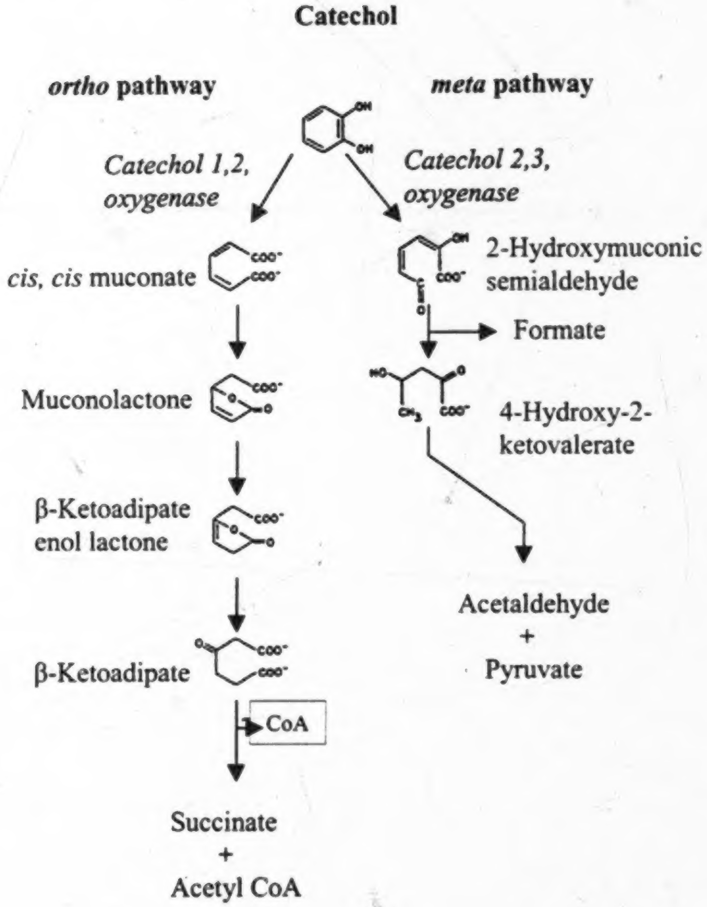

Catechol
ortho pathway
meta pathway
OH
OH
Catechol 1,2, oxygenase
Catechol 2,3, oxygenase
cis, cis muconate
COO⁻
COO⁻
2-Hydroxymuconic semialdehyde
Formate
Muconolactone
4-Hydroxy-2-ketovalerate
HO
CH₃
β-Ketoadipate enol lactone
Acetaldehyde
+
Pyruvate
β-Ketoadipate
CoA
Succinate
+
Acetyl CoA

Figure 1.1. *Ortho* and *meta* cleavage pathways for the degradation of catechol. Adapted from Dagley et al. (1960); Burlage et al. (1989)

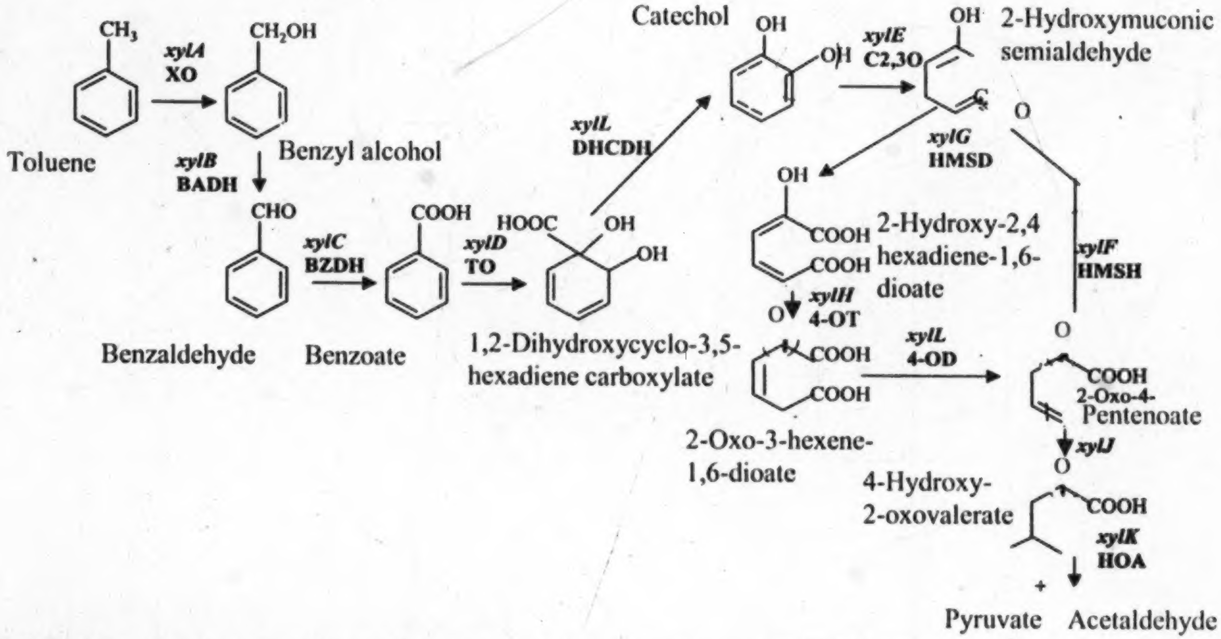

Toluene
xylA
XO
Benzyl alcohol
xylB
BADH
Benzaldehyde
xylC
BZDH
Benzoate
xylD
TO
1,2-Dihydroxycyclo-3,5-hexadiene carboxylate
xylL
DHCDH
Catechol
xylE
C2,3O
2-Hydroxymuconic semialdehyde
xylG
HMSD
2-Hydroxy-2,4 hexadiene-1,6-dioate
xylH
4-OT
2-Oxo-3-hexene-1,6-dioate
xylL
4-OD
xylF
HMSH
2-Oxo-4-Pentenoate
xylJ
4-Hydroxy-2-oxovalerate
xylK
HOA
Pyruvate
Acetaldehyde

Figure 1.2. Pathway for the degradation of toluene encoded by *Pseudomonas* plasmid pWW0. The specific degradation genes are listed in italics and the abbreviation of the enzymes that they encode are in bold face letters (for complete names of the enzymes, see Table 1.1). Adapted from Nakazawa and Yokota (1973): Burlage et al. (1989).

Table 1.1. Genes and enzymes of the TOL degradative pathway. Adapted from Burlage et al. (1989).

Gene	**Enzyme or function**	**Abbreviation**
xylA	Xylene oxygenase	XO
xylB	Benzyl alcohol dehydrogenase	BADH
xylC	Benzaldehyde dehydrogenase	BZDH
xylD	Toluate oxygenase	TO
xylE	Catechol 2,3-oxygenase	C 2,3O
xylF	2-Hydroxymuconic semialdehyde hydrolase	HMSH
xylG	2-Hydroxymuconic semialdehyde dehydrogenase	HMSD
xylH	4-Oxalocrotonate isomerase	4-OT
xylI	4-Oxalocrotonate decarboxylase	4-OD
xylJ	2-Oxopent-4-enoate hydratase	OEH
xylK	Dihydroxycyclohexadiene carboxylate dehydrogenase	DHCDH
xylX,Y	Terminal oxidase	-
xylR	Regulation	-
xylS	Regulation	-

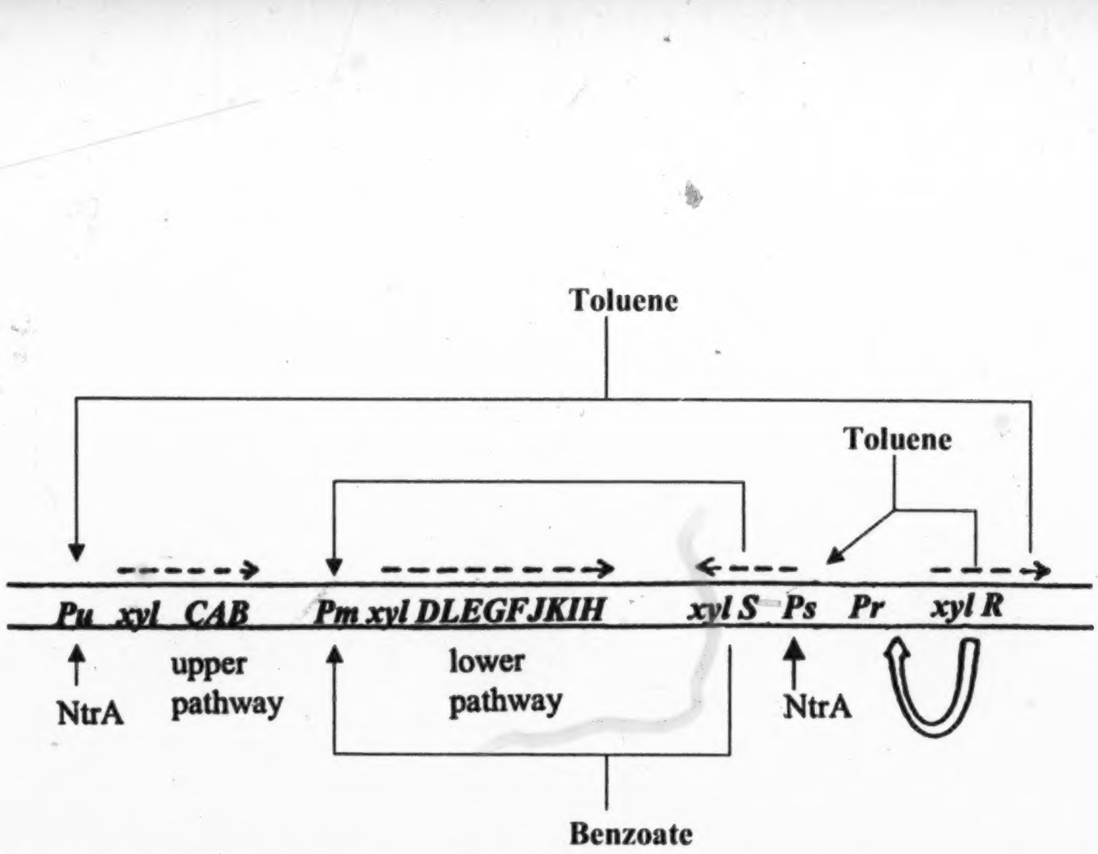

Toluene
Toluene
Pu xyl CAB
Pm xyl DLEGFJKIH
xyl S Ps Pr xyl R
NtrA
upper pathway
lower pathway
NtrA
Benzoate

Figure 1.3. Proposed model for gene regulation in pWW0. Broken arrows indicate the direction of transcription. Thin continuous arrow represent activation of the indicated operons; open arrows represent repression. Promoters requiring NtrA protein are identified. In this figure, toluene combines with the XylR protein to affect two promoters, Pu and Ps. Increased production of XylS can then activate Pm. Alternatively benzoate in the presence of the constitutive amount of XylS is able to activate Pm. The *xylR* gene is shown to be an autorepressor. Adapted from Ramos et al. (1987); Inouye et al. (1987) and Burlage et al. (1989).

pathway. In cells carrying the genes for both the pathways, only the *meta* pathway is expressed. In variants that have lost the *meta* pathway, the *ortho* pathway functions and such cells then outgrow the others. Such 'benzoate-cured' variants, i.e. TOL plasmids that have lost the *meta* pathway due to growth on benzoate, can arise either by loss of the whole plasmid or by the spontaneous excision of the specific contiguous 40 kb segment encoding the catabolic region (Bayley et al., 1977). Duetz and van Andel (1991) showed that mutants lacking TOL catabolic genes also arose when *P. putida* mt-2 harboring TOL was grown in chemostat cultures with succinate and under sulphate, ammonium or phosphate limitations. They speculated that the presence of partially deleted plasmids rather than plasmid-free mutants might occur because cryptic plasmid-encoded functions could be advantageous under the given conditions, whereas maintenance of the complex catabolic pathway might be a severe burden for the host under conditions that are growth-limiting. The spontaneous loss of this region, coupled with the loss of degradative capacity, led Chakrabarty et al. (1978) to propose that a transposon may be associated with the TOL plasmid, effecting intraplasmidic recombination between two direct repeat sequences, which flank the degradative pathway (Meulien et al., 1981). A 56 kb and 17 kb region on plasmid pWW0 were subsequently shown to be transposons (Jeenes and Williams, 1982; Tsuda and Iino, 1987).

Many other TOL plasmids have been described, most of which have similar biochemical pathways and regions of strong DNA homology to pWW0 (Keil et al., 1985; Whited et al., 1986; Williams and Worsey, 1976). TOL-like plasmids are usually found in *Pseudomonas* species, as gene expression seems to be limited to or favored by

Pseudomonas and related species (Chatterjee and Chatterjee, 1987). Only the catabolic region has been well studied in the TOL plasmid.

Bradley and Williams (1982) determined that pWW0 encodes a thick, flexible, constitutively expressed pilus and that pWW0 is naturally derepressed for transfer in its original *P. putida* host. Bradley et al. (1980) reported that TOL transferred better on a surface than in liquid. Smets et al. (1993) investigated the conjugal transfer of a TOL plasmid using *P. putida* PAW1 as donor and *P. aeruginosa* PAO1162 as recipient and reported that transfer rates of TOL are sufficient to maintain TOL in a dense microbial community without selective pressure. Christensen et al. (1996) described that TOL plasmid transfer on semi-solid surfaces occurred mainly during a short period after initial contact of donors and recipients indicating that the spread of the TOL plasmid is limited in static, but viable cultures.

1.5. NAH7 plasmid

Dunn and Gunsalus (1973) first reported the presence in *P. putida* strain G7 (ATCC 17485) of the naphthalene catabolic plasmid NAH, encoding a naphthalene-(Nah$^+$) and salicylate-(Sal$^+$) utilizing phenotype. They also reported that the Nah$^+$ Sal$^+$ phenotype of *P. putida* PpG7 (NAH7) could be transferred by conjugation to both *P. putida* PpG7 and PpG1 derivatives. This plasmid was later renamed NAH7 by Farrell (1980). Yen and Serdar (1988) reviewed the literature describing naphthalene catabolic plasmids and stated that they belong either to the IncP7 or IncP9 group and that they all encode a homologous upper pathway. In addition, most of them specify the same pathway for salicylate degradation. Sanseverino et al. (1993) stated that NAH7 prevents its host from mineralizing other polyaromatic hydrocarbons.

Genes for naphthalene metabolism are localized on the 83 kb self-transmissible NAH7 plasmid in two gene clusters contained within a 30 kb region of NAH7 (Yen and Gunsalus, 1982). The naphthalene catabolic pathway and the enzymes involved are presented in Figure 1.4 and Table 1.2. The salicylate derived from napthalene is oxidized to catechol, which is the ring fission substrate for the *meta* pathway or the *ortho* pathway (Yen and Serdar, 1988). The *nah* catabolic genes of plasmid NAH7 are found on a defective class II transposon (Tsuda and Iino, 1990). Yen and Serdar (1988) in their review stated that the first operon encodes the conversion of naphthalene to salicylate (upper pathway) and the second operon codes for the oxidation of salicylate via the catechol *meta*-cleavage pathway to acetaldehyde and pyruvate (lower pathway) as well as encoding the regulatory gene product which controls both operons with salicylate as the inducer. Catechol is metabolized by the same biochemical pathway in TOL and NAH7. The structural organization of the gene cluster for this *meta*-pathway is highly conserved in the two plasmids and there is a high degree of sequence homology in the regions responsible for *meta*-pathway and in the plasmid replication and transfer functions (Lehrbach et al., 1983; Harayama et al., 1987).

Duncan et al. (1995) studied the effects of naphthalene on the fitness of *P. putida* in microcosms containing sterile soil. They found that naphthalene had a negative effect on fitness and the plasmid was disadvantageous in the absence of naphthalene. The rate of horizontal transmission was a function of the product of the densities of the donor and recipient and the greatest development of transconjugants occurred in microcosms that

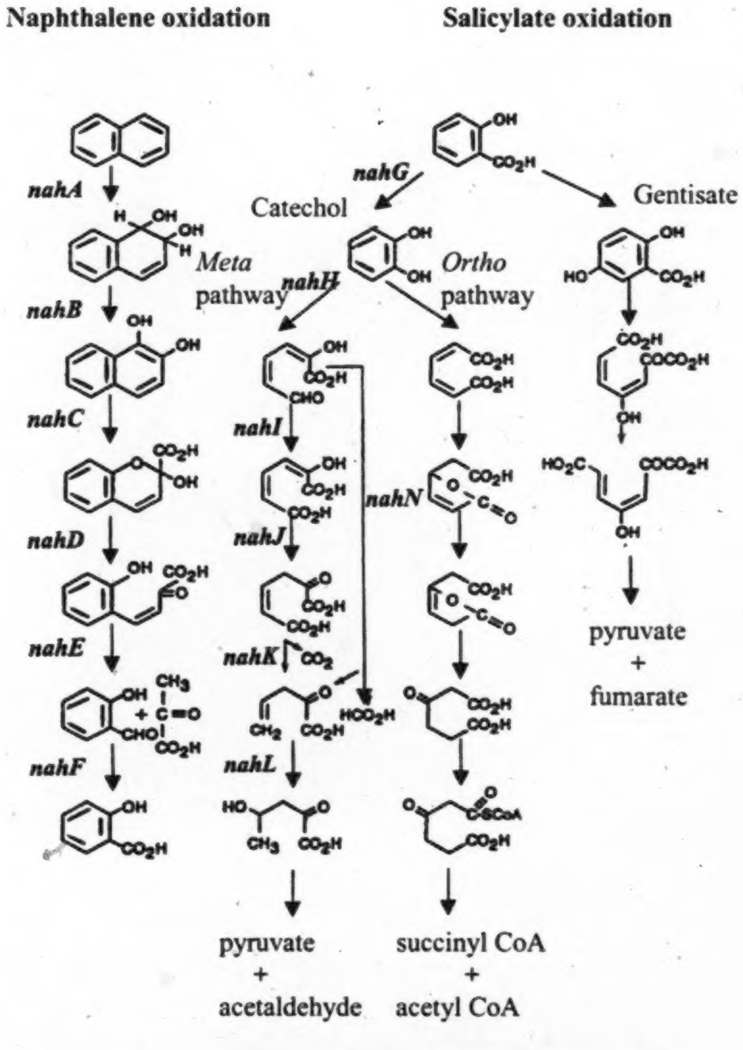

Naphthalene oxidation
Salicylate oxidation
nahA
nahB
nahC
nahD
nahE
nahF
nahG
Catechol
Gentisate
Meta
pathway
nahH
Ortho
pathway
nahI
nahJ
nahK
CO_2
nahL
nahN
HCO_2H
pyruvate
+
fumarate
pyruvate
+
acetaldehyde
succinyl CoA
+
acetyl CoA

Figure 1.4. Naphthalene and salicylate catabolic pathways in *Pseudomonas*. The genes are listed adjacent to the arrows. See Table 1.2.for enzymes encoded by the genes. Adapted from Yen and Serdar (1988).

Table 1.2. Genes and enzymes of the naphthalene degradative pathway. Adapted from Yen and Serdar (1988).

Genes	**Enzymes**
nahA	naphthalene dioxygenase
nahB	*cis*-naphthalene dihydrodiol dehydrogenase
nahC	1,2-dihydroxynaphthalene oxygenase
nahD	2-hydroxychromene-2-carboxylate isomerase
nahE	2-hydroxybenzalpyruvate aldolase
nahF	salicylaldehyde dehydrogenase
nahG	salicylate hydroxylase
nahH	catechol oxygenase
nahI	2-hydroxymuconic semialdehyde dehydrogenase
nahJ	2-hydroxymuconate tautomerase
nahK	4-oxalocrotonate decarboxylase
nahL	2-oxo-4-pentenoate hydratase
nahN	2-hydroxymuconic semialdehyde hydrolase

did not contain naphthalene. Herrick et al. (1997) tried to provide field evidence for horizontal transfer by detecting the 16S rRNA sequences and naphthalene dioxygenase iron-sulfur protein (*nahAc*) genes of various naphthalene degrading bacteria isolated from contaminated sites. Lack of phylogenetic congruence of the *nahAc* and 16S rRNA genes indicated recent horizontal transfer, possibly as a direct or indirect consequence of pollutant contamination. The plasmid sizes varied from 70 to 88 kb and the authors suggested that plasmid modification after transfer might have been effected by transposons.

1.6. Plasmid transfer in soil

Plasmid transfer on agar plates or in broth in the laboratory has been widely studied in *E. coli* and other related bacteria but studies on plasmid transfer in soil are scarce (Schilf and Klingmuller, 1983; Trevors and Oddie, 1986; Trevors and Starodub, 1987; van Elsas et al., 1988). Conjugation has been demonstrated between strains of *E. coli* (Trevors and Oddie, 1986) and *Pseudomonas* (Lattorre et al., 1984) in soil, *Burkholderia cepacia* in soil slurries (Walter et al., 1989) and *Klebsiella* in the rhizosphere (Talbot et al., 1980).

Soil is a discontinuous environment composed of irregularly distributed, isolated and structurally diverse particles, all of which affect the distribution of introduced organisms, their survival and subsequently the transfer of genetic material between microbial populations. Biological and physiochemical characteristics of the soil also play a major role in gene transfer (Scott et al., 1992). A frequent approach to the determination of the environmental conditions that favour conjugation has been to study plasmid transfer in laboratory matings and to investigate the effects on such matings of variations in physical parameters that are relevant to the environment (Fernandez-Astorga et al., 1992). Microcosms simulating important elements of the environment can be designed to evaluate horizontal transfer of catabolic plasmids. Natural systems are inherently complex and it is difficult or impossible to design laboratory experiments that account for the multiple interactions in even the simplest of them. A pragmatic approach is to use a model to identify and describe the main factors that determine system behavior at one level and then to make predictions about a higher-level system on the basis of these specific assumptions (Knudsen et al., 1988). The use of microcosms is fundamental to research in microbial ecology to gain an understanding of plasmid-mediated gene dispersal and the extent to which it affects the microbial community structure and dynamics in the natural soil environment. However, a "standard soil microcosm" does not exist and therefore conclusions based on comparisons made between different microcosm studies under even slightly different environmental conditions must be made carefully (Hill and Top, 1998).

Some factors affecting plasmid transfer in soil include: soil type; availability of nutrients and energy sources; availability of oxygen; temperature, humidity and pH; transmissibility and host range of the plasmid; density of donors and recipients; duration of mating period; persistence of donor cells during the mating period; competition between donors, recipients, transconjugants and indigenous microflora in non-sterile soil (Klingmuller et al., 1990). Other factors to be considered include the distribution of donor and recipient cells in the soil, light-dark cycles and selection of the most suitable media for enumeration of donors, recipients and transconjugants.

Conjugation is affected by temperature. For example, Gauthier et al. (1985) found an increase in transfer frequency between 25° and 30°C from a marine pseudomonad to *E. coli in vitro*, an effect due to the temperature. In sterile soil and water inoculated with donors and recipients, plasmid transfer was found by Trevors and Oddie (1986) to occur at temperatures as low as 5°C.

Soil pH is also an important factor in affecting conjugal transfer. Krasovsky and Stotzky (1987) confirmed that maximum transfer frequencies occurred at a soil pH of approximately 7. Richaume et al. (1989) studied transfer of pBLK1-2 from *E. coli* to *Rhizobium fredii* and they observed maximum transfer frequencies at a soil pH of 7.25 with the additional parameters of clay addition of 15%, an organic matter addition of 5%, a soil moisture content of 8% and a soil incubation temperature of 28°C. They also observed that cell-to-cell contact was enhanced by the addition of organic matter addition and resulted in an increased transfer frequency at the 5% level.

Though sterile soil does not constitute an ideal microcosm, Trevors et al. (1987), stated that it represented a compromise between strict laboratory conditions and *in situ* experiments. Klingmuller (1991) monitored transfer of pEA9, a 200 kb self-transmissible plasmid, between nitrogen-fixing *Enterobacter* strains, and found that addition of Luria broth or sucrose to non-sterilized soil elicited strong cell propagation and plasmid transfer. It was observed that at 22°C, pH 5.2, 15.5% moisture, loose soil, 2 x 10^7 inoculated donor and recipients, pEA9 transfer was observed only in sterilized soils and not in non-sterile soils. Neilson et al. (1994) showed that the transfer of pJP4, a large 2,4-dichlorophenoxyacetic acid (2,4-D) catabolic plasmid, occurred under ideal growth conditions in nonsterile soil but that the transfer frequency is greatly reduced compared with transfer in sterile soil or on solid agar medium. In non-sterile soil, transfer can only be detected if the donor cells and the recipients survive in sufficient numbers during the period under study. This is dependent on their fitness in the soil environment and their ability to compete with the indigenous microflora for nutrients and energy (Klingmuller et al., 1990). Conjugation between pseudomonads in soil is significantly stimulated by the presence of plant roots but it is unclear if the effect is due to root exudates, root (hair) surfaces, or both (van Elsas et al., 1988). Hirsch and Spokes (1994) demonstrated that a Tn*5*-marked symbiotic plasmid survived and remained viable in soil for over six years. They stated that it should not be assumed that introduced inoculants disappear after a few years and that bulk movement of soil during cultivation is likely to be a major route for the spread of introduced microorganisms. Daane et al. (1997) reported transfer of the catabolic plasmid pJP4 between physically isolated bacteria in nonsterile soil, using burrowing earthworms as a biological factor to facilitate cell-to-cell contact. Transfer of the catabolic plasmid pWW0-EB62, encoding degradation of ethylbenzoate, between introduced strains in sterilized soil with and without addition of ethylbenzoate was only detected when the donor and recipient strains were of the same species (Ramos-Gonzalez et al., 1991).

All transconjugants observed *in situ* are not a direct result of transfer occurring in the soil as there will be subsequent survival, growth and die-off of transconjugants in competition with donors, recipients and indigenous oil microorganisms. Increases in transconjugant numbers with time are also likely to result from the replication of the initial transconjugants. Therefore, the sampling interval after inoculation will influence the transconjugant numbers observed and any subsequent interpretation of these counts (Hill and Top, 1998).

There has been very little information pertinent to horizontal transfer of catabolic plasmids in microcosms. This could be due to the limited interest in the exciting aspect of microbial ecology, difficulty in working with and detecting transfer of large catabolic plasmids or due to the excessive interest bestowed on antibiotic resistance plasmid transfer. The controlled application of bioremediative bacteria with plasmid borne degradative genes to contaminated sites could lead to bioaugmentation. Neilson et al. (1994) stated that biaugmentation strategies uniformly suffer from a lack of information regarding the stability of introduced genes and corresponding physiological traits in the environment. Fulthorpe and Wyndham (1991) observed transfer of the 3-chlorobenzoate plasmid pBRC60 from *Alcaligenes* sp. strain BR60 to indigenous aquatic microorganisms in freshwater flowthrough mesocosms.

Hirsch et al. (1997), based on their work and also citing Atlas and Bartha (1993), proposed that organic contaminant compounds might provide selective pressure for genetic adaptation in field sites, because in many habitats carbon is the limiting factor for growth and metabolism of heterotrophic microorganisms. Even a low frequency of transfer might enable the successful establishment of transconjugants especially if there is selective pressure. DiGiovanni et al. (1996) studied pJP4 transfer from *A. eutrophus* JMP134 to indigenous soil recipients. They indicated that gene transfer is an effective means of bioaugmentation and the survival of the introduced organism is not a prerequisite for biodegradation that utilizes introduced catabolic genes. The kinetic events that govern conjugal transfer might influence their contribution to community adaptation to environmental changes. Although the selective pressure for a plasmid-encoded gene might be very small and, therefore, would predict the washout of the plasmid from a community, horizontal transfer within the community might be effective in maintaining the plasmid in the community (Smets et al., 1993).

Duncan et al. (1995) suggested that repeated rounds of horizontal transfer followed by intense competition in soil microcosms might facilitate the selection of strains well suited for bioremediation. Duetz and van Andel (1991) suggested that the proliferation of strains harboring catabolic plasmids might be limited if the extra carbon and energy gain was not sufficient to compensate for this burden. For a rational assessment of the extent of gene transfer in the environment, it is also necessary to know the persistence, survival, competition, nutrition, stress and the physiological state of the introduced bacteria (van Veen et al., 1997).

1.7. Use of a biomarker (Green Fluorescent Protein) to detect plasmid transfer

A simple and generally effective method to identify a particular microorganism is to mark it with a specific genetic marker or a series of markers that are unlikely to occur naturally at a detectable frequency in the environment. Marked gene products can be detected by measurement of their biological activity or immunological reactivity (Saunders et al., 1989). Biomarkers could serve as valuable tools to detect transfer of catabolic plasmids because positive selection techniques such as using PAH to select for the incoming plasmid will not be required. Pickup (1991) reviewed methods for monitoring DNA transfer including DNA-DNA hybridization and simple plating on selective media. However, molecular techniques require sample preparation and manipulation, and antibiotic resistant cells are naturally present in soil, limiting the use of certain resistance markers in detecting gene transfer. Moreover, these methods cannot provide direct *in situ* information about conjugal transfer. Luciferase (*luxAB*) genes have limitations also, owing to their requirement for a substrate and naturally luminescent bacteria are present in the environment.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has proved to be a valuable tool in biology. Prasher et al. (1992) cloned the gene for GFP from *A. victoria* to understand the mechanisms of light generation in the luminescent jellyfish organ. The GFP expressed in prokaryotic and eukaryotic cells is capable of producing a strong green fluorescence when excited by blue light. In the jellyfish, GFP is activated in a calcium-dependent manner when Ca^{2+} binds another bioluminescent protein, aequorin, which transfers energy indirectly to trigger the release of green light. This energy transfer can be mimicked experimentally by simple exposure of GFP to standard long-wave ultraviolet light from a conventional fluorescence microscope or a hand-held UV source (Prasher, 1995; Misteli and Spector, 1997).

This protein (GFP) is fluorescent and unlike Lux, does not require the addition of other substrates, or cofactors. Because GFP fluorescence requires no additional gene products from *A. victoria*, chromophore formation is not species-specific and occurs either through the use of ubiquitous cellular components or by autocatalysis. GFP does not interfere with cell growth and function (Chalfie et al., 1994) and its expression can be detected *in situ*. The strength of GFP as a marker lies in the detection of individual cells without any processing in a nondestructive manner. In contrast it is not possible to distinguish between growth of transconjugants and gene transfer when selective media are used for transconjugant detection. For example, the use of green fluorescent protein has removed some of the constraints imposed by the need to select for particular genes (Dahlberg et al., 1998; Christensen et al., 1998). Transfer of plasmid pBF1, tagged with the *gfp* gene controlled by a *lac* promoter that is down-regulated in the donor cell by a chromosomal repressor (lacI^q), was studied in seawater. Expression occurred under nutrient-limited conditions after transfer to several defined recipients on surfaces and in bulk seawater, as measured on a single-cell level without limiting detection of gene transfer to the culturable fraction of bacteria (Dahlberg et al., 1998). The authors also proposed that the GFP method complemented traditional detection of transconjugants with selection plates and that this nondisruptive technique was well-suited to studies of surface communities where the spatial distribution of transconjugants can be recorded.

With GFP, it is possible to get a conservative measure of the transfer event. Stretton et al. (1998) used GFP to tag and investigate gene expression in marine bacteria, and visualized and monitored gene expression in living single cells *in situ* and in real time. Leff and Leff (1996) used GFP to monitor survival of genetically engineered bacteria in aquatic environments and stated that microscopic determination was more accurate than cfu enumeration. Christensen et al. (1996) used GFP as a biomarker for monitoring the horizontal transfer of TOL plasmid in *P. putida* and observed the distribution of cells active in conjugation. Burlage et al. (1996) studied the movement of GFP-labeled strains through a homogeneous medium and reported that bacteria can be accurately tracked using their fluorescence. However, the variability of GFP expression in different species and under various environmental conditions and interference by other fluorescent particles or bacteria in a particular system may make it difficult to utilize GFP-derived fluorescence to quantitate cell numbers (Bloemberg et al., 1997).

1.8. Objectives of this thesis

Although TOL and NAH7 plasmids have been studied for a long period, there has been very limited work on their transfer to other recipients. There is a need to obtain more information on the horizontal transfer of catabolic plasmids like TOL and NAH7 to understand conjugal transfer of these plasmids and the importance of plasmid transfer in bioremediation.

The objectives of this thesis were: to develop a model system to study conjugal transfer of TOL and NAH7 plasmids; to examine conjugal transfer of TOL and NAH7 in microcosms subjected to different physical conditions; to use green fluorescent protein as a biomarker to track the movement of TOL and NAH7; to evaluate the amount of mobile catabolic DNA in soils contaminated with polycyclic aromatic hydrocarbons by attempting exogenous and endogenous isolations of plasmids from such soils. I hypothesised that different physicochemical and biological factors would influence transfer of TOL and NAH7.

In this thesis, I report progress towards a better understanding of factors influencing horizontal transfer of the catabolic plasmids TOL and NAH7 in sand microcosms, such as temperature, pH, parental strain density and ratios and selective pressure. This work also shows exciting glimpses into the various deletion and insertion events that occur during transfer of these large plasmids. GFP has been used as a biomarker for detecting plasmid transfer.

Chapter 2

Materials and Methods

2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Tables 2.1, 2.2 and 2.3. Plasmid pWW0 is referred to as TOL throughout this thesis. Rifampicin resistant mutant strains were generated by UV mutagenesis and kanamycin resistant mutants by transposon mutagenesis. Transposon mutagenesis was performed by electroporating the plasmid pGS9::Tn*5*Km^r into the desired recipients as described in Section 2.13.3.2.

2. 2. Media and chemicals

The compositions of all media, chemicals and buffers used are listed alphabetically arranged subsections in Appendices B and C. In mating experiments, transconjugants were selected on mineral salts medium (MM) with nutritional selection (0.5 mM *m*-toluate or vapors from naphthalene crystals) to select for the incoming plasmid plus antibiotic (Rif or Nal) for donor counterselection.

Total heterotrophs were enumerated using ¼ strength Plate count agar (PCA, Difco, Detroit, MI), Bushnell-Haas (BH) with PAH or MM with PAH as carbon source were used to isolate degraders. The different carbon sources used included phenanthrene (98% purity), anthracene (99.9%), fluoranthene, chrysene, pyrene and fluorene (98%) (Aldrich Chemical Company, WI), naphthalene (Sigma, St Louis, MO) and *m*-toluate (Pfaltz & Bauer Inc, Stanford, CN). Pigment-producing pseudomonads were also grown on Pseudomonas agar F and Pseudomonas agar P media (Difco, Detroit, MI). Spray plates or agar plates were prepared as described in Appendix B.

Table 2.1. Characteristics of bacterial strains used in this work

Strains	Phenotype[a]	Plasmid[b]	References
P. putida strains			
mt-2 (ATCC 33015)	Cm^r, Sp^r, Sm^r, Tc^r, Ap^r *m*-tol$^+$	pWW0 (TOL)	Worsey and Williams (1974)
PpG7 ATCC 17453	Cm^r, Sp^r, Sm^r, Tc^r, Ap^r, Nah$^+$	NAH7	Yen and Gunsalus (1982)
KT2440	Cm^r, Ap^r, restriction$^-$ modification$^-$	-	Mamool et al. (1986) Gift from W. Finnerty, U. Georgia
KT2440Rif	Cm^r, Ap^r, Rif^r	-	This study
Variants of KT2440 KTT1Y	**Derived from KT2440** Cm^r, Ap^r, Rif^r; *m*-tol$^+$	TOL	This study
KTN4	Cm^r, Ap^r, Rif^r, Nah$^+$	NAH7	This study
KTΔT	Cm^r, Ap^r, Rif^r; *m*-tol$^+$	ΔTOL	„
KTFN2	Cm^r, Ap^r, Rif^r; Nah$^+$	+NAH7, NAH7	„
KTFNF2	Cm^r, Ap^r, Rif^r; Nah$^+$	NAH7, Two variants of +NAH7	„
KTF4	Cm^r, Ap^r, Rif^r, Nal^r; Nah$^+$	NAH7	„

KTF7	Cm^r, Ap^r, Rif^r, Nal^r; Nah$^+$	+NAH7	"
KT2440Sm	Cm^r, Ap^r, Sm^r	-	This study
Other *Pseudomonas* strains			
P. alcaligenes DM201Rif	Sm^r, Ap^r, Nal^r, Km^r, Rif^r, Nah$^-$	-	Gift from W. Finnerty, U. Georgia
P. putida NCIB 10643	Rif^r, Nah$^-$	-	"
P. putida MFY Rif	Rif^r, Nah$^-$	-	This study
P. aeruginosa AK1401	Rif^r, *m*-tol$^-$, Nah$^-$	-	Berry and Kropinski (1986)
P. aeruginosa AK1414	*m*-tol$^-$; Nah$^-$	-	"
Pseudomonas sp. Devon	Rif^r, *m*-tol$^-$	-	This study

[a] *m*-tol$^+$, growth utilizing *m*-toluate as C source
Nah$^+$, growth on naphthalene as C source
m-tol$^-$ or Nah$^-$, non-utilization of *m*-toluate or naphthalene as C source
Ap, Cm, Rif, Km, Sm, Sp, Nal, Tc (see Abbreviations) – antibiotic resistance exhibited by the organism

[b] pWW0, 115 kb TOL plasmid
NAH7, 83 kb plasmid
ΔTOL, Deletion in TOL plasmid (Section 3.3)
+NAH7, Insertion in NAH7 plasmid (Section 3.4.1)

Table 2.2. Accessories- Strains and plasmids used

E. coli strains	phenotype	Plasmid	References
JE 2571	Ap^r, Tc^r, Km^r (plasmid); Sm^r	RP1	Gift from L. Frost, U. Alberta
EN24	Sp^r		,,
WT	Su^r, Sm^r	R3006	Gift from D. Taylor, U. Alberta
6354	Ap^r, Tc^r	RP4:: Km^s	Bradley et al. (1980)
HB101	Km^r	pRK2013	Gift from K. Roy, U. Alberta
HB101	Km^r	pGS9-Tn*5*	Selvaraj and Iyer (1983)

[a] Ap, Tc, Km, Su, Sm, Sp -antibiotic resistance exhibited by the organism

Table 2.3. Strains and plasmids used in studies with Green Fluorescent Protein

Organisms and plasmids	**Phenotype**[a]	**Comment**	**Reference**
E. coli (pSM1373)	Km^r	Mini Tn*5* transposon vector. *gfp* gene under the control of T7 promoter	Christensen et al. (1996) Gift from S. Molin, Lyngby
E. coli (pSM1384)	Km^r	Mini Tn*5* vector. Delivery of bacteriophage T7 RNA polymerase gene	„
P. putida SM1396	Rif^r	chromosomal integration of T7 RNA polymerase	„
P. putida SM1396 CT7	*m*-tol$^+$, TOL::*gfp*; Rif^r Nal^r ; Km^r	GFP expressing strain; TOL mutagenized by *gfp*	This study
P. putida SM1396 AN7	Nah$^+$, NAH7::*gfp*; Rif^r, Nal^r ; Km^r	GFP expressing strain; NAH7 mutagenized by *gfp*	„
P. putida KT2440 Sm CT7	*m*-tol$^+$, TOL::*gfp*; Cm^r, Ap^r, Sm^r ; Km^r	non-GFP expressing strain; TOL mutagenized by *gfp*	„
P. putida KT2440 Sm AN7	Nah$^+$, NAH7::*gfp*; Cm^r, Ap^r, Sm^r ; Km^r	non-GFP expressing strain; NAH7 mutagenized by *gfp*	„

[a] *m*-tol$^+$- growth on *m*-toluate as C source
Nah$^+$ -growth on naphthalene crystals
GFP-green fluorescent protein
Cm, Ap, Sm, Km, Nal, Rif- Antibiotics used for selection

For plasmid isolation, cultures were grown in 50 ml Luria Bertani (LB) medium (Miller, 1972) in 125 ml flasks incubated at 28°C. Antibiotic resistance of the isolates was determined on LB + antibiotic plates. The antibiotics used were Cm (30 μg/ml), Km (50 μg/ml), Tc (150 μg/ml), Ap (40 μg/ml), Sp (100 μg/ml), and Rif (100 μg/ml) (Sigma St Louis, MO). For estimating metal resistances, an agar dilution method was used. Copper and mercury as copper sulphate and mercuric chloride respectively were incorporated into mineral medium + glucose (2g/l) and 100 μl of the microorganisms were spotted on the plates (Nieto et al., 1989) to determine metal resistances of the isolates (as described in Appendix B). Growth was scored after 2 days incubation at 28°C.

In the experiments with the GFP, media used included FABC (Christensen et al., 1996) and LB with iron ethylenediamine tetraacetic acid (Sigma, St Louis, MO) to reduce background fluorescence from *Pseudomonas*.

2.3 Transformation

Transformation studies were performed to find out if transformation could act as a mechanism for acquisition of the TOL or the NAH7 plasmids by *P. putida* KT2440Rif^r. The source of transforming DNA was prepared as either a crude cell lysate (1 ml), of *P. putida* mt-2 (TOL) or *P. putida* PpG7 (NAH7), by the addition of lysis solution (Appendix B) or as isolated TOL or NAH7 plasmid DNA (approximately 1μg; for plasmid isolation refer Section 2.11.2). Overnight LB grown cultures of *P. putida* KT2440Rif^r and the source of DNA were deposited onto sterile 0.22 μm (2 cm diameter) pore size filter disc (Millipore, Mass), placed face up on a LB plate and incubated for 18 h at 28°C.

The recipient was also made competent by adopting the procedure used in the development of electrocompetent cells (Section 2.13.3.1) and transformation carried out. The bacteria on the filter were resuspended in 5 ml of 0.85% sterile saline by vigorous agitation in an 18 x 150 mm test tube, using a vortex mixer. The transformation mixture was then plated without diluting on selection plates i.e. MM + Rif + naphthalene or *m*-toluate and incubated at 28°C. Separate, parallel donor and recipient controls were performed.

2. 4. Bacterial mating procedures

Mating procedures were carried out to determine plasmid linked phenotypes and horizontal transfer. Three methods were used in this project to study transfer of catabolic plasmids: mating of donor and recipient cells on filter membranes incubated on solid medium; mixing of donors and recipients and spotting the cells directly on agar plates; and incubating mixed broth cultures of donors and recipient cells as described in Sections 2.4.1 to 2.4.3. All incubations of mating mixtures were carried out under non-selective conditions (i.e. without antibiotic or carbon source selection) and all experiments were performed in duplicate.

2. 4. 1. Filter matings

Cultures of recipients and donors grown overnight in LB on a rotary shaker at 200 rpm (28°C) were used for filter mating. One hundred microlitres each of donor and recipient cultures were mixed in a 1 cc sterile syringe (Becton Dickinson, Franklin Lakes, NJ) with an extra 100 µl of LB medium and filtered using a Millipore Swinnex W/O Filter unit onto a sterile 0.22 µm pore size filter disc (Millipore, Mass), placed face up on a LB plate and incubated for 18 h at 28°C. The bacteria on the filter were resuspended in 5ml of 0.85% sterile saline by vigorous agitation in an 18 x 150 mm test tube, using a vortex mixer to interrupt mating. The cell suspension was serially diluted in 10 mM phosphate buffer (pH 7) and plated on appropriate selection media. Separate, parallel donor and recipient controls were performed.

2. 4. 2. Plate mating

One hundred microlitres each of overnight donor and recipient cultures (ca. 10^9 cells/ ml) grown in LB were mixed gently in a microfuge tube by vortex, then 100 µl sterile LB was added, and the entire suspension was spotted on LB plates using a micropipette. Parallel donor and recipient controls were also performed. Plates were incubated ca. 18 h at room temperature. After incubation, the cells were recovered from the surface of the plate using a sterile inoculation needle and suspended in 4 ml of 0.85% saline by vortex. Care was taken to ensure that the agar layer underneath was not scraped when the cells were removed to avoid carryover of the LB medium. Ten fold serial dilutions were performed in phosphate buffer and 0.1 ml plated on selection plates. Two-fold dilutions rather than decimal dilutions were carried out when TOL plasmid transfer was studied to avoid problems arising due to too many colonies to count.

2. 4. 3. Broth mating

One millilitre each of overnight cultures of donor and recipient (grown in LB) were inoculated into 5 ml LB and incubated on a tube roller (200 rpm) and in a stationary condition overnight at room temperature (ca. 25°C). Ten-fold serial dilutions were performed as described in section 2. 4. 2. and plated on selection plates.

2. 5. Discrimination between filter mating and mating on selection plates

Mating between the donor and the recipient might occur on the selection plate itself (i.e. after removal from the conjugation filter), and transconjugants might be a result of true filter mating or an artefact of subsequent mating on selection plates. It is essential to distinguish between these two possibilities as this might lead to inflated estimates of conjugal transfer. Pre-incubating selection plates at 4°C and including nalidixic acid for donor counterselection reduces the number of transconjugants, compared with plating at 20°C and 27°C (Smit and van Elsas 1990). Therefore, this method was used to test the potential for mating on selection plates in this study.

P. putida strains PpG7 (NAH7) and KT2440Rif^r were used as the plasmid donor and recipient respectively. Donors and recipients were grown overnight in LB broth (50 ml culture in 125 ml flasks) to a density of ~10^9 cells/ml, estimated by viable plate count. Standard filter matings (Section 2.4.1) on LB plates were incubated at 28°C for 18 h. Two sets of selective plates (MM + Rif + naphthalene) were used. One set was preincubated at 4°C for ca. 18 h and the other kept at room temperature. Controls were performed by plating donors or recipients separately on selective plates to check for spontaneous mutants. Donor and recipient cells were mixed and immediately plated on the transconjugant selection plate to assess whether mating occurred on the selection plate. The 4°C preincubated plates, after inoculation, were maintained for another 3 h at 4°C before further incubation at room temperature for 24 h.

The presumptive transconjugants were replica plated on MM + Rif + Naphthalene and tranconjugants confirmed as outlined in Section 2.11.1 and 2.11.2.

2. 6. Conjugal transfer kinetics

Studying the conjugal transfer kinetics of TOL and NAH7 would offer exciting insights into rate of transfer of these large plasmids and differentiate between waves of horizontal and vertical transfers.

Donors and recipients were grown overnight in LB medium in shake flasks (50 ml in 125 ml flasks) at 28°C. Replicate aliquots of 50 µl each of donor and recipient cells were spotted on LB agar using the procedure described in Section 2.4.2. Every 200 min to a total of 2800 min (46.6 h), one spot was recovered from the agar surface, suspended in 0.85% saline, serially diluted in phosphate buffer and plated on selection medium. All plates were incubated at 28°C and the experiment was performed in duplicate. Separate, parallel donor and recipient controls were performed. Donors and recipients grown separately and mixed at the time of sampling were also plated on selection plates as controls. A repeat experiment was performed from 0 to 800 min and sampled every 100 min.

2. 7. Donor:recipient ratios

A higher donor to recipient ratio could influence conjugal transfer frequencies. Standard plate matings were carried out (Section 2.4.2) using 1:1, 1:5 or 1:10 ratios of donor: recipient cells (D:R), grown to a cell density of ca. 10^9cells/ml overnight in LB. Plates were incubated at 28°C for 24 h and transconjugants were selected on appropriate selection medium.

2.8. Conjugal transfer in sand microcosms

Microcosms simulating important elements of the environment can be designed to evaluate horizontal transfer of catabolic plasmids. It is difficult to design laboratory experiments that account for all the multiple interactions that might exist in natural ecosystems. However, the use of microcosms is fundamental to research in microbial ecology to gain an understanding of plasmid mediated gene dispersal.

P. putida strains PpG7 (NAH7) and ATCC33015 (TOL) were used as plasmid donors and KT2440Rif^r *m*-tol$^-$, nah$^-$, was used as the recipient. Acid-purified sand (BDH Chemicals, Toronto, ON) used for the microcosms was prewashed with phosphate buffer (pH 7) and air dried at 55°C to stabilize the pH. Each microcosm consisted of 50 g (dry weight) of sand in a 150 ml beaker stoppered with a foam plug and twice sterilized by autoclave (wrapped cycle) before use. The soil was moistened with sterile mineral medium to give final moisture content of 20 – 23 % (wet weight basis) and a pH of 7. To recover transconjugants, 1 g of soil was taken from each of three locations in the microcosm, pooled, mixed vigorously by vortex in 4 ml of 0.1% sodium pyrophosphate, serially diluted in phosphate buffer and plated on appropriate transconjugant selection plates.

2.8.1. Validation of sampling technique

This method was performed to ascertain that the techniques used to sample the sand microcosm yielded representative subsamples: a trial was performed wherein an entire microcosm was sacrificed for comparison with a parallel 1 g sampling.

The donor used was *P. putida* strains PpG7 (NAH7) and KT2440Rif^r the recipient, at a 1:1 donor to recipient ratio. Microcosms in beakers were prepared as described. Parallel glass dilution bottles stoppered with rubber stoppers, containing 50 g acid purified sand were also performed. The moisture content was adjusted to 20% (wet weight basis) and inoculation of donor and recipient performed as described in Section 2.8.3. Fifty millilitres of 0.1% sodium pyrophosphate was added to the bottle, which was shaken vigorously for 2-3 min. This was treated as the undiluted sample. The experiment was incubated in parallel with the beaker microcosms at 25°C. Selection plates used were MM Rif + Nah and the plates were incubated at 28°C.

2.8.2. Effect of selective pressure

When NAH7 plasmid transfer was studied, selective pressure was provided *in situ* by addition of naphthalene to the microcosms. This was supplied, either as crystals aseptically sprinkled and mixed with the sand, or dissolved in diethyl ether and added to the microcosm to a final concentration of 7.5 mmoles/ml medium in the sand. The ether was allowed to evaporate overnight in the fumehood before the microcosm was inoculated.

The initial cell density of donor and recipient was 10^9 cells/ml (estimated by viable plate count). Overnight cultures grown in LB were pelleted by gentle centrifugation (4000 rpm for 2 min in an Eppendorf 5415C centrifuge) and pellet resuspended in 0.85% saline to avoid any residual LB medium being introduced in the microcosms. Two millilitres of cell suspension was inoculated into each donor and recipient control microcosm. In the mating mixture microcosm, 2 ml of donor and 2 ml of recipient suspension were inoculated on the surface without mixing. All the microcosms were then supplemented with 8 ml of mineral medium and 5 ml of 10 mM phosphate buffer (pH 7.0) to give a final moisture content of 23% on a wet weight basis, then incubated at 28°C. For microcosms prepared without selective pressure, mineral medium was substituted with LB (8 ml).

When the TOL plasmid was used, *m*-toluate was provided in mineral medium at a final loading rate of 5 mmoles/ml of medium.

All incubations were carried out at room temperature for 24 hours. Transconjugants were recovered, serially diluted and plated on appropriate selection plates (Section 2.8). The experiments were performed twice in triplicate.

2.8.3. Effect of temperature

Temperature is another important factor that affects conjugal transfer. To study NAH7 transfer, naphthalene crystals (~2g) were added to the microcosms directly by mixing with the sand before inoculation. Cultures were pre-grown in LB to ca. 10^9

cells/ml and 2 ml each of donors and recipients, mixed before addition, were added to the centre of the microcosms, which were incubated at 10, 20, 25 or 30°C.

To study TOL plasmid transfer, a 1:5 donor to recipient ratio was used at incubation temperatures of 10, 20, 25, 30 or 35°C. These microcosms were supplied with *m*-toluate dissolved in mineral medium to a final loading rate of 5 mmoles/ml of medium. Transconjugants were recovered and selected on MM + Rif + naphthalene or *m*-toluate. Presumptive transconjugants were replica plated on the appropriate transconjugant selection plates incubated at 28°C, and hydrocarbon degrading colonies (90 colonies each) were screened for the presence of plasmids. Donor and recipient control microcosms were maintained separately. The experiment was performed twice in triplicate.

2.8.4. Effect of pH

P. putida strains PpG7 (NAH7) and KT2440Rif^r were used as donor and recipient respectively. Matings were performed in sand microcosms provided with naphthalene crystals (~ 2 g) sprinkled onto the surface of the microcosm. The pH of the microcosm was adjusted using MM buffered at different pHs using phosphate buffer (Breznak and Costilow, 1992). The pH of the mineral medium was determined, using a pH meter (Fisher Accumet Model 620), after sterilization by autoclave to account for changes in pH due to precipitation of phosphates. The different conditions tested were pH 5.8, 6.3, 6.6, 7.0, 7.2 and 7.6 (see Appendix B). Donor and recipient cells grown overnight in LB and washed in 0.85% saline, were used in a 1:1 ratio for the experiment and microcosms were incubated at 25°C for 18 h. Donors, recipients and transconjugants were enumerated from mating microcosms on appropriate selection plates. The experiment was performed twice in duplicate.

2.8.5. Disturbed and compacted sand microcosms

The structure of the substratum in the microcosm might also interfere with conjugal transfer, as it would affect cell-cell contact of donors and recipients. Therefore, the effect of competition on plasmid transfer was studied as described. After addition of naphthalene crystals (~2 g), MM and donor and recipient inocula, the sand was stirred up thoroughly using a sterile spatula to give a disturbed microcosm.

Conversely, after compacting (by pressing the sand down aseptically), MM was added and donor and recipient cells were inoculated into the centre of the microcosm (Section 2.8.2). Naphthalene crystals were sprinkled on top of the sand to provide a compacted/undisturbed sand microcosm.

The microcosms were incubated at 25°C for 24 hours, after which transconjugants, donors and recipients were enumerated on appropriate selection plates. The experiment was performed twice in duplicate.

2.9. Competition Experiment

Single donor-single recipient interactions do not usually occur in nature. A wide variety of interactions, including competition between microflora components, might abound and this would play an important role in conjugal transfer.

P. putida PpG7(NAH7) was used as the donor and *P. putida* KT2440Rif^r as the competitor with *P. putida* DM201Km^r or *P. aeruginosa* AK1414Km^r as the recipients (refer Table 2.1). The experiment was carried out according to the procedure adopted for selection pressure experiment (Section 2.8.2) and performed once in triplicate.

2.10. Authentic soil microcosms

Sand, as a relatively inert substrate, does not offer the dynamism of a soil microcosm. Agricultural soil was collected from Ellerslie Research Station (University of Alberta) by quartering. The soil was sterilized by autoclave for 20 min in glass beakers three times with a time interval of two days after each autoclaving cycle to kill all endospores. Soil sub-samples were then serially diluted and plated on LB to confirm sterility.

The soil experiments were performed at 25°C as described previously (Section 2.8.2) with a starting inoculum density of 10^8 cells/g of wet soil. Fifty grams of autoclaved and dry soil was used in 150 ml glass beakers. A 1:5 donor to recipient cell ratio was adopted. The methodology adopted was similar to that described in Section 2.8.2. Separate donor and recipient controls were maintained. The experiment was performed in duplicate and cell densities were expressed as cfu /g of soil on a wet weight basis.

2.11. Molecular techniques

2.11.1. Confirmation of transconjugation

Success of conjugal transfer was confirmed by carrying out plasmid preparations to screen for the presence of the transferred plasmid. Plasmid transfer to the recipient strains was first assessed by testing the presumptive transconjugants for all plasmid-associated resistance and degradative phenotypes using the appropriate selective medium. After this test, the presumptive transconjugants were then replica plated on appropriate selective medium using sterile toothpicks. Approximately 50 presumptive transconjugants were then inoculated into 5 ml LB in 18 x 150 mm test tubes, grown to turbidity overnight in a tube roller at 28°C and screened for the presence of plasmids by a modification of the method of Kieser (Kieser, 1984).

Stability of the acquired plasmids was assessed by repeated transfers of isolated transconjugants in LB broth without selection pressure and monitored by subsequent plasmid DNA screening. As further verification, restriction digestion analysis was performed (see Section 2.11.2, 2.11.5 and 2.11.6 for detailed method).

2.11.2. Isolation of plasmid DNA by Kieser's method

Small-scale preparations of plasmid DNA were carried out by using a modification of the alkaline lysis method (see Appendix C for reagents). This method was found to be especially suitable for isolation of large catabolic plasmids.

Approximately 1.5 ml of an overnight grown culture was transferred to an Eppendorf tube and cells were harvested by microcentrifugation at 13,000 rpm in a benchtop Eppendorf centifuge (Eppendorf 5415C) for about 30 sec. The supernatant was removed and the cell pellet was suspended in 500 µl of lysis buffer (Appendix C) by mixing on a vortex mixer. Then 250 µl of lysis solution (Appendix C) was added and the tubes were inverted quickly to ensure that the lysis solution was thoroughly mixed. The tubes were incubated in a water bath at 55°C for 20 min till a clear lysate was obtained. The tubes were then cooled to room temperature, 500 µl of potassium acetate (pH 4.8) was added and the tubes were gently mixed by inversion to disperse the solution through the bacterial lysate. The tubes were then incubated on ice for 5 min and the cell debris was removed by microcentrifuge (13,000 rpm for 5 min). The supernatant was transferred to a new tube, 250 µl of phenol:chloroform was added, vortexed gently and the aqueous phase was separated from the organic phase by microcentrifugation at 13,000 rpm for 5 min. The aqueous phase was transferred to a new tube and a similar wash was performed with chloroform. Finally the aqueous phase was transferred to a new Eppendorf tube. DNA was precipitated by the addition of an equal volume of isopropanol and incubation overnight at –20°C or for 1 h at –70°C. DNA was collected by centrifugation at 13,000 rpm and the pellet was washed with 1 ml of 70% v/v ethanol. The pellet was then allowed to air-dry for about 20 min, DNA dissolved in 20 µl of sterile ddH$_2$O and stored at -20°C. This method could be easily scaled up to isolate plasmid DNA from larger culture volumes.

2.11.3. Isolation of plasmid DNA by Casse's method

This method, originally used for the isolation of large plasmids in *Rhizobium meliloti* (Casse et al., 1979) was adopted to isolate large plasmids from the environment (see Appendix C for reagents).

Overnight cultures (10 ml) in pre-weighed sterile Teflon Oak Ridge tubes (25 ml) were harvested at 12,100 g for 10 min in a Sorvall RC-5B Refrigerated superspeed centrifuge, washed once with 50 mM Tris: 20 mM Na$_2$EDTA (pH 8) and repelleted. The pellet weight was adjusted to no more than 0.1 g, as too much of pellet results in viscous solutions and poor preparations. The pellet was then resuspended in 0.5 ml of 50:20 TE.

Lysing solution (4% SDS in 50:20 TE, pH 12.4) was freshly made; 9.5 ml of the lysing solution was added and mixed gently but thoroughly. The tubes were incubated at 55°C for 10 min and neutralized with 1 ml of 2 M Tris-HCl (pH 7). Solid NaCl (0.33 g) was then added to precipitate the chromosomal DNA and gently mixed to dissolve the salt. The tubes were incubated at 25°C for 30 min and 10 ml of equilibrated phenol was added. After centrifugation at 8000 g, 8 ml of the upper aqueous phase was transferred to a new tube using a wide mouth pipette. The DNA was then precipitated by the addition of 1/10 the volume 3 M sodium acetate (pH 5.2). Standard isopropanol precipitation and 70% ethanol washes were then performed and DNA was stored at -20°C.

2.11.4. Isolation of chromosomal DNA

Chromosomal DNA was isolated based on a modification of the Marmur procedure. Overnight culture (~10 ml) was centrifuged in a Teflon Oak Ridge tube at 12, 100 g for 5 min in a in a refrigerated superspeed centrifuge (Sorvall RC-5B). The cell pellet was then resuspended in 5 ml sterile saline EDTA, re-pelleted at 12,100 g for at least 5 min and resuspended in 2.5 ml sterile saline. Two hundred microlitres of 25% (w/v) SDS was added, mixed thoroughly and incubated at 60°C for 10 min. After cooling to room temperature, 600 µl of 5 M sodium perchlorate and 3 ml of freshly made 24:1 isoamyl alcohol:chloroform was added and mixed by gentle inversion and shaking for 30 min in a shaker at 150-200 rpm. The tubes were then centrifuged at 12,100 g for 5 min to separate layers. The upper layer was transferred to a new tube and layered with 4 ml 95% ethanol. The DNA was then spooled using a Pasteur pipette hook by gentle swirling motion. The DNA was rinsed in 95% ethanol, gently dissolved in 0.1 to 0.5 ml of sterile ddH_2O and stored at –20°C.

2.11.5. Restriction Endonuclease Digestion of DNA

Restriction digestion analysis was performed to determine the digestion profiles of the different plasmids used in this project and to verify the plasmid content of the transconjugants.

Plasmid DNA or chromosomal DNA were digested (Sambrook et al., 1989) using common restriction endonucleases including *Eco*RI, *Bam*HI, *Sma*I and *Not*I, purchased from Boehringer-Mannheim. Digestions were performed according to manufacturer's instructions using the provided buffer, restriction endonucleases and containing < 1 µg of DNA per reaction. The reaction mixture after appropriate incubation was analysed by gel electrophoresis as outlined in Section 2.11.6.

2.11.6. Gel electrophoresis of DNA

DNA or DNA digests were separated on 0.8-1.8% w/v agarose gels in TAE buffer (Appendix C). Gel electrophoresis was then performed in a horizontal submerged system (Bio-Rad). Before loading the sample on the gel, loading buffer (Appendix C) was mixed with the DNA. The size of the DNA fragments were determined by comparison with molecular weight standards: λDNA digested with *Hind*III or *Bst*EII (Boehringer Mannheim or New England Biolabs). Using known plasmids, a circular plasmid molecular weight marker generated was used to determine sizes of undigested plasmids isolated from environmental isolates. Electrophoresis was carried out at constant voltage

(80 V), and agarose gels (*Ultrapure* agarose from Gibco BRL, Gaithersburg, MD) were stained with ethidium bromide (10 µg/ml in dH_2O), and DNA was visualized on a UV transilluminator. The image was captured using an imager (Appligene Oncor, Gaithersburg, MD) attached to a Mitsubishi Video Copy Processor Model P68U.

Vertical gels were also run to determine the presence of plasmids from environmental isolates. Agarose gels (0.6-0.7% w/v) were cast in the vertical gel apparatus with TBE buffer (Appendix C). Twenty-five microlitres of sample DNA with loading dye was loaded on to the gel. All gels were run at 80 V for 3-4 h.

2.12. Gel extraction of DNA and Hybridization

This procedure was performed to determine the origin of the insertion fragment on the +NAH7 plasmid. This involved purifying the inserted fragment and using the fragment to probe wild type NAH7 plasmid by Southern transfer and hybridization. Southern transfer and hybridization was also performed to determine if the plasmids from environmental isolates harbored degradative genes (see Section 2.13.3). For detailed methodology see Section 2.12.1-2.12.4.

2.12.1 Gel extraction of insertion fragment from +NAH7 DNA

The digested DNA was run on a 1% agarose gel at 90 V to get well separated bands. The DNA band corresponding to the insertion fragment on the NAH7 plasmid was excised from the gel and purified using the Geneclean kit (BIO 101 Inc, Vista, CA), based on the adherence of DNA to glass milk, according to the manufacturer's instructions. Care was taken to excise only the insertion fragment. Once excised from the gel, the fragment was purified by phenol/chloroform extraction and ethanol precipitation.

2.12.2. Southern Transfer

Southern transfer was performed using the transfer protocol of Rigaud et al. (1987). After electrophoresis, the gel was placed in a glass dish and the DNA was depurinated by soaking in 0.25 M HCl. Two washes of 15 to 30 min each were performed by slow gentle shaking on a platform shaker at room temperature. The DNA was then denatured by performing two washes of 20 min each in denaturing solution (0.5 N NaOH, 1.5 M NaCl). The gel was then rinsed in dH_2O, placed in neutralizing solution (1 M ammonium acetate, 0.02 N NaOH) and washed twice for 30 min each by gentle shaking on a platform shaker. The gel was then transferred to the capillary blot transfer apparatus. This was set up using a glass baking dish filled with 1.5 L of the neutralizing solution, with a glass plate on top, using a piece of Whatman 3MM paper as a wick placed across the glass plate, with ends resting in the reservoir of neutralizing solution. The gel was placed on the saturated wick and surrounded with Saran Wrap. A piece of pre-wetted 0.45 micron Hybond ^{TM}-N nylon membrane (Amersham Life Sciences, England) was placed on the gel, followed by three sheets of Whatman 3MM paper and a ~5 cm stack of paper towels after squeezing out any air bubbles. A glass plate with a 1 kg weight was placed on top of the paper towels and transfer allowed to proceed overnight. The next day the apparatus was dismantled, the wells of the gel were marked with a pencil for later identification and the membrane was removed. The DNA was then cross-linked to the membrane using a UV-cross-linker (Bio-Rad GS Gene Linker ^{TM}).

2.12.3. Preparation of probes by random primer labeling

The random primer method (Sambrook et al., 1989) was used by V. M. Anoop to prepare ^{32}P-labelled DNA fragments to probe wild type NAH7 and its derivatives. Approximately 50 ng of purified DNA in 10 µl ddH_2O was denatured by boiling for 10 min and then rapidly cooling on ice. The following components were then added to the denatured DNA: 3 µl dNTP mix (0.5 mM each of dATP, dGTP, dTTP; Boehringer Mannheim), 5 µl [$\alpha^{32}P$]-dCTP (10 µCi/µl, Amersham, 2 µl 10X hexanucleotide mix (Boehringer Mannheim) and finally 1 µl Klenow fragment of DNA polymerase (2 units/µl, Boehringer Mannheim). The reaction was left overnight at room temperature (25°C), after which time the reaction was stopped by heating to 65°C for 10 min. Unincorporated nucleotides were removed by adding 3 µl Blue Dextran (in 0.5 M EDTA, pH 8.0) and passing the mixture through a Sephadex G-50 (in TE buffer) column. The probe was denatured by heating to 100°C for 5 min, before being used for hybridization.

2.12.4. DNA:DNA Hybridization

The membrane from Southern Transfer was placed in a glass hybridization bottle. One millilitre of pre-warmed hybridization solution was added per 10 cm^2 of membrane along with 10 $\mu l/cm^2$ of sonicated salmon sperm DNA (stock concentration of 50 µg/ml from a 10 mg/ml solution which had been denatured by boiling for 5 min). Pre-hybridization was allowed to occur for at least 4 hours at 55°C, with shaking in a shaker. After pre-hybridization, the labeled and denatured probe (Section 2.12.3.; $\sim 3 \times 10^6$ cpm) was added along with fresh hybridization solution. Hybridization was allowed to occur overnight at 60°C. After hybridization, the membrane was washed, first in 2X SSC, 0.1% w/v SDS solution twice for 15 min at room temperature and followed by a 30 min wash in 1X SSC, 0.1% SDS at 45°C. A more stringent wash was not carried out. The membrane was then placed on a Whatman 3MM filter paper, wrapped in Saran Wrap and autoradiography performed at –70°C for up to 4 days using a Kodak X-OMAT™ AR X-ray film. The above procedure was carried out by V. M. Anoop.

2.13. Construction of TOL::*gfp* or NAH7::*gfp* mutants

Transposon mutagenesis, when the transposon is borne on a suicide plasmid, is dependent on conjugation. Selection for the transposon means that only conjugation events that are followed by a transposition event would be detected. The gene for GFP was available cloned into Tn*5* carried on a non-self transmissible suicide vector, pSM1373 (Christensen et al., 1996; refer Table 2.3). Using three factor mating (2.13.1) or electroporation (2.13.3), the Tn*5*::*gfp* was mobilized into recipients containing the TOL or NAH7 plasmid. Integration of the Tn*5*::*gfp* cassette into the catabolic plasmid tagged the plasmid with both Km resistance and the potential to express GFP. The *gfp* is under the control of the T7 promoter and therefore is expressed only in recipients having T7 RNA polymerase. The Tn*5*::*gfp* tagged catabolic plasmids were selected on appropriate selection plates to ensure that the catabolic region was not disrupted or lost during mutagenesis. Different strategies were adopted to transposon mutagenize the TOL and the NAH7 plasmids with Tn*5*::*gfp*, as described in the following three sections. All the media used for selection is described in Appendix B.

2.13.1. Three factor mating

In this method, *P. putida* KTT1Y strains harboring TOL was used as the recipient. *E. coli* 6354 containing the helper plasmid (RP4) was used to mobilize the suicide vector pSM1373 with the *gfp* gene from *E. coli* into a recipient with the TOL plasmid. A 1:5:1 donor:recipient:helper cell ratio was tested to increase the frequency of Tn*5* mutagenesis. Similar matings were carried out to transposon mutagenize the NAH7 plasmid except that *P. putida* KTN4 and naphthalene crystals were used instead of *P. putida* KTT1Y and *m*-toluate.

The presumptive TOL::*gfp* or NAH7::*gfp* was mated into a recipient cell, *P. putida* SM1396Nal with the with the T7 RNA polymerase gene for expression of green fluorescence as outlined in Figure 2.1. This method was adapted from Christensen et al. (1996). A nalidixic acid (100 µg/µl) resistant strain of *P. putida* SM1396 was used to provide a better means of donor counterselection. All matings were carried out on LB plates and all plates incubated at 28°C.

2.13.2. Retromobilization of suicide vector with Tn*5*::*gfp* by helper plasmid and subsequent transposon mutagenesis of degradative plasmid.

This strategy is based on the simple assumption that the probability of transposon mutagenesis of the degradative plasmid i.e., TOL and NAH7, can be increased by first carrying out a retromobilization of the suicide vector using the helper plasmid and subsequent transfer of the helper plasmid and pSM1373::*gfp* into a recipient harboring a degradative plasmid. The methodology implemented is outlined in Figure 2.2.

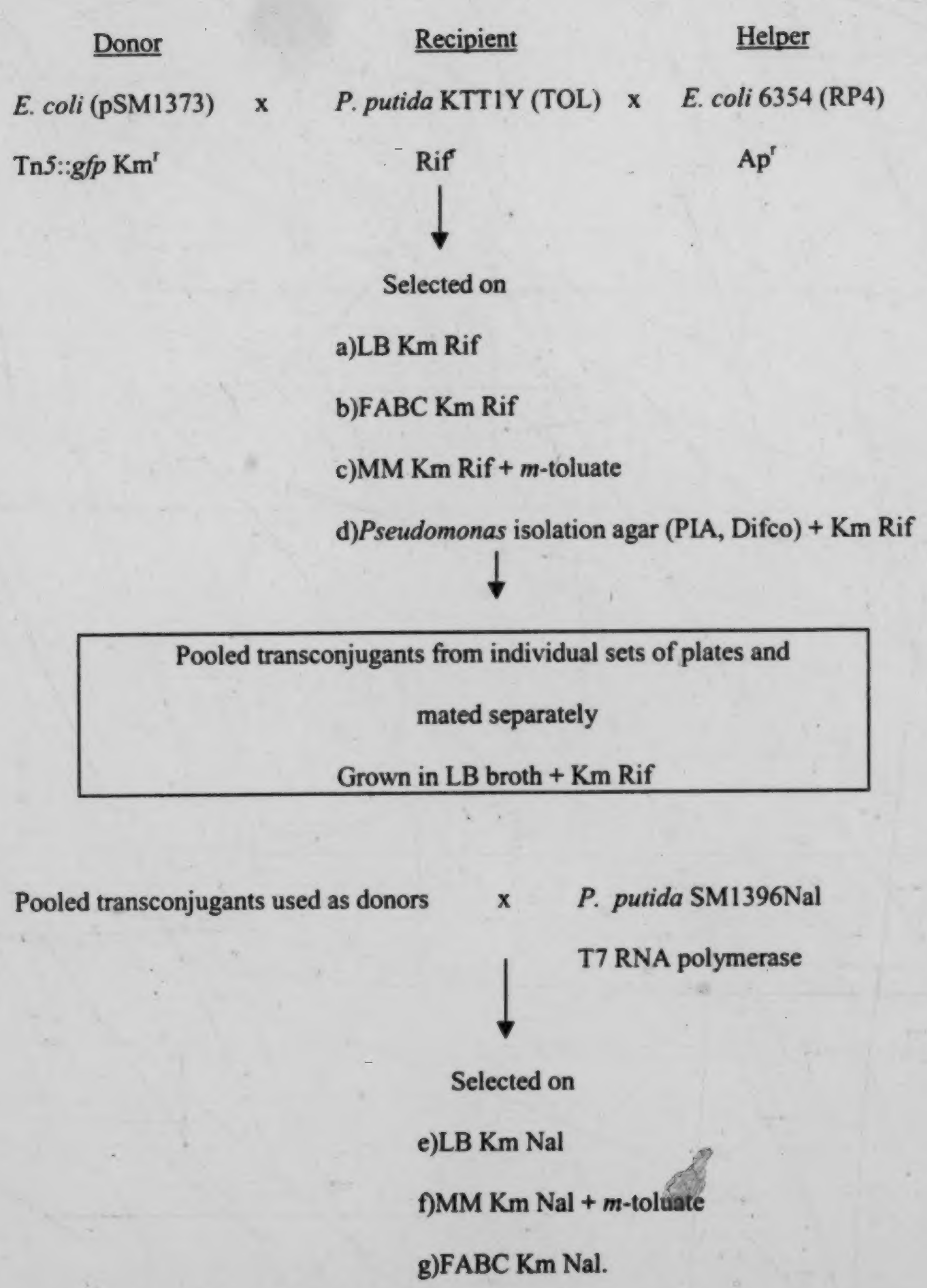

Donor
Recipient
Helper
E. coli (pSM1373)
x
P. putida KTT1Y (TOL)
x
E. coli 6354 (RP4)
Tn*5*::*gfp* Km^r
Rif^r
Ap^r
Selected on
a)LB Km Rif
b)FABC Km Rif
c)MM Km Rif + *m*-toluate
d)*Pseudomonas* isolation agar (PIA, Difco) + Km Rif
Pooled transconjugants from individual sets of plates and
mated separately
Grown in LB broth + Km Rif
Pooled transconjugants used as donors
x
P. putida SM1396Nal
T7 RNA polymerase
Selected on
e)LB Km Nal
f)MM Km Nal + *m*-toluate
g)FABC Km Nal.

Figure 2.1. Schematic diagram showing three factor mating for transposon mutagenesis of TOL plasmid with Tn*5*::*gfp* and creation of expression strain for GFP detection

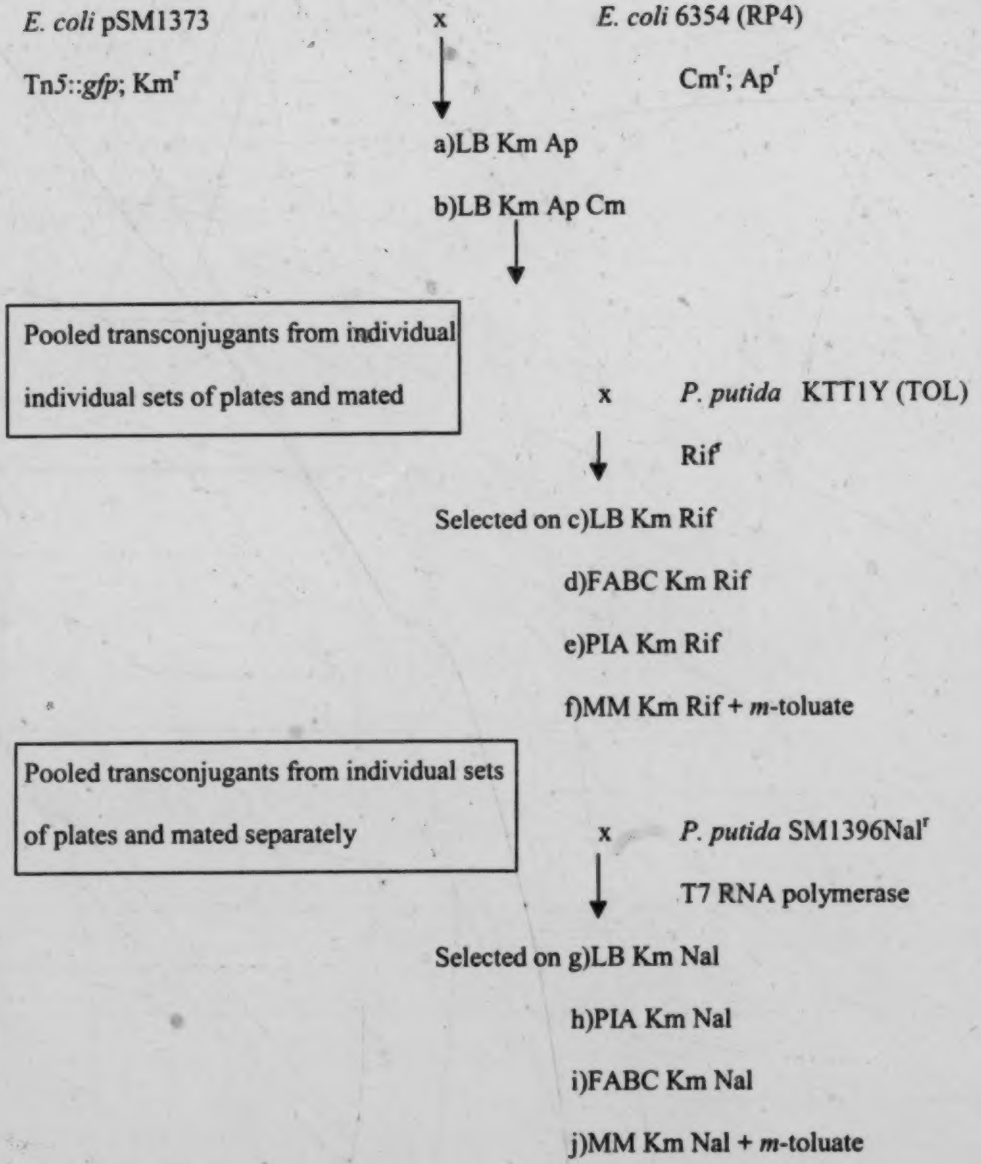

E. coli pSM1373
x
E. coli 6354 (RP4)
Tn*5*::*gfp*; Km^r
Cm^r; Ap^r
a)LB Km Ap
b)LB Km Ap Cm
Pooled transconjugants from individual individual sets of plates and mated
x
P. putida KTT1Y (TOL)
Rif^r
Selected on c)LB Km Rif
d)FABC Km Rif
e)PIA Km Rif
f)MM Km Rif + *m*-toluate
Pooled transconjugants from individual sets of plates and mated separately
x
P. putida SM1396Nal^r
T7 RNA polymerase
Selected on g)LB Km Nal
h)PIA Km Nal
i)FABC Km Nal
j)MM Km Nal + *m*-toluate

Figure 2.2. Retromobilization of suicide vector by RP4, subsequent transposon mutagenesis of TOL plasmids, and expression of TOL::*gfp* in *P. putida* SM1396.

2.13.3. Electroporation

DNA can be introduced into cells using electroporation by creating transient micropores in the cell with a strong pulse of electric current. Electrocompetent cells of *P. putida* KTT1Y(TOL) or KTN4(NAH7) were prepared (2.13.3.1) and the vector with Tn*5*::*gfp* was electroporated into the competent cells (Figure 2.3).

2.13.3.1. Electrocompetent cell preparation

The uptake of DNA by electroporation depends on the competence of the cells. Cultures were grown overnight to OD of 0.35-0.5 and 10 ml was harvested in sterile Teflon Oak Ridge tubes (25 ml tubes) at 12,100 g for 10-15 min at 4°C (Sorvall RC-5B Superspeed centrifuge). The pelleted cells were then washed with an equal volume of pre-chilled 10% glycerol, centrifuged as before and washed again with ½ the volume 10% glycerol. The cells were finally resuspended in 40 µl of 10% glycerol/ml of culture. The cells were dispensed in 40 µl aliquots into Eppendorf tubes in a dry ice-ethanol bath, chilled 5 min and then stored at –70°C.

2.13.3.2. Electroporation procedure

About 500 ng of donor DNA and 40 µl of thawed electrocompetent recipient cells were mixed in cold Electroporation Cuvettes Plus (0.2 cm gap; BTX Genetronics Inc, CA). This suspension was electroporated at the following setting: voltage, 2.5 kV; resistance 400 Ω; and capacitance, 25 µFd using Gene Pulser II (Bio-Rad), yielding a 12.5 kV/cm field strength. After electroporation, cells were allowed to recover in 960 µl

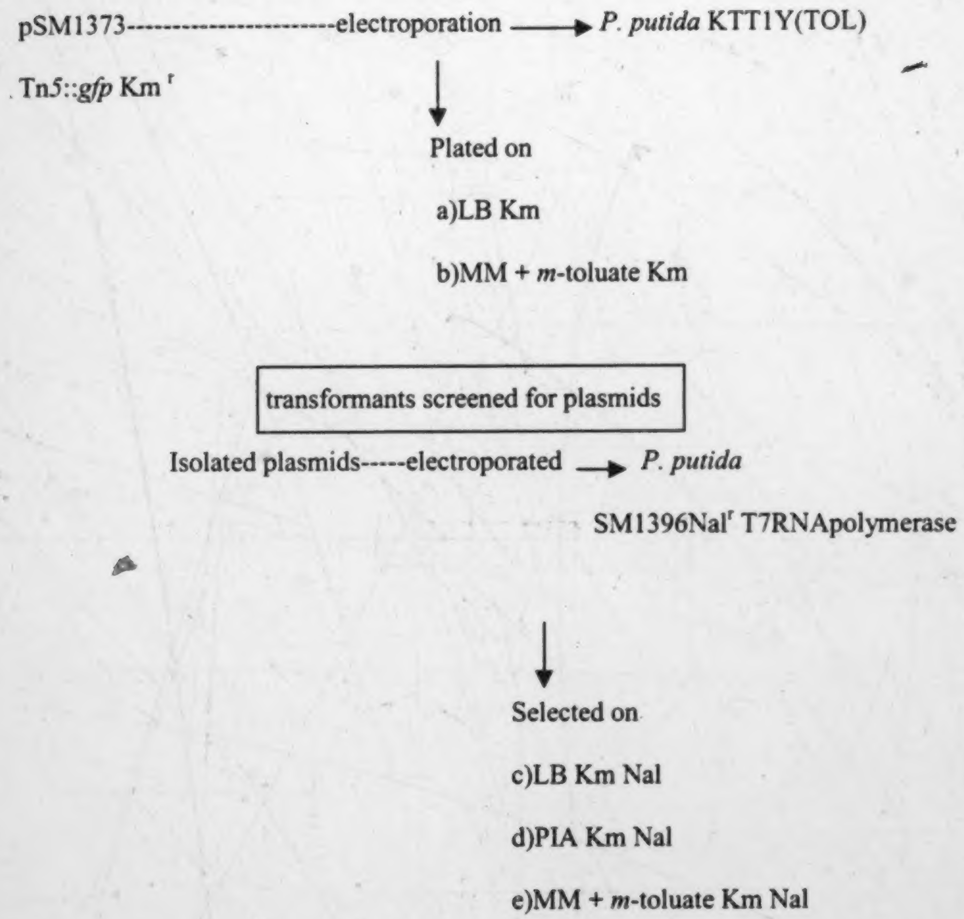

pSM1373----------------------electroporation → *P. putida* KTT1Y(TOL)
Tn*5*::*gfp* Km^r
Plated on
a)LB Km
b)MM + *m*-toluate Km
transformants screened for plasmids
Isolated plasmids-----electroporated → *P. putida*
SM1396Nal^r T7RNApolymerase
Selected on
c)LB Km Nal
d)PIA Km Nal
e)MM + *m*-toluate Km Nal

Figure 2.3. Schematic diagram showing electroporation, transposon mutagenesis and generation of GFP positive *P. putida* SM1396

SOC medium (Appendix B) for ~6 hours at 28°C in a tube roller. The electroporated cells were plated onto appropriate selection medium. Controls included plating of electrocompetent cells on to selection medium without exposure to DNA, and subjecting electrocompetent cells to an electric impulse without transforming DNA and subsequent plating.

2.13.4. Verification of TOL::*gfp* and NAH7::*gfp* mutants

TOL::*gfp* and NAH7::*gfp* were introduced by electroporation into a recipient lacking the T7 RNA polymerase gene to show that they did not express gfp, but were still *m*-tol$^+$ or Nah$^+$. Then the tagged plasmids were moved by electroporation back into a recipient having the T7 RNA polymerase gene to demonstrate GFP production by fluorescence. GFP-expressing *P. putida* SM1396Nal^r harboring TOL::*gfp* or NAH7::*gfp* were used as plasmid donors. A *P. putida* KT2440Sm^r strain was used as the first recipient and *P. putida* SM1396 Nal^r T7 RNA polymerase was used as the final recipient.

The presumptive TOL::*gfp* plasmid was isolated from GFP-expressing *P. putida* SM1396Nal^r, and electroporated into *P. putida* KT2440Sm^r which lacks the T7 RNA polymerase gene (using the methods described in Section 2.13.3.2). Plasmid transfer was detected on LB Km Sm and MM + *m*-toluate Km Sm media but the cells did not express GFP. *P. putida* KT2440Sm^r (TOL::*gfp*) transformants were pooled, plasmid DNA was isolated and electroporated into *P. putida* SM1396Nal^r. Plasmid transfer was detected by plating on LB Km Nal and MM + *m*-toluate Km Nal media and GFP production was observed as described (Section 2.13.5.1).

The same methods (Figure 2.3) were adopted for generating NAH7::*gfp* mutants except that naphthalene crystals instead of *m*-toluate were added to selective plates to select for acquisition of the NAH7 plasmid.

After each electroporation, plasmid DNA isolated from selected transformants was digested with *Eco*RI. DNA fragments were analyzed on a 1% agarose gel (see Section 2.11.5) to confirm Tn*5*::*gfp* integration on TOL and NAH7 plasmids.

2.13.5. GFP Detection methods

Two methods were tested for detection of cell fluorescence on agar plates due to GFP expression which are described below (Section 2.13.5.1.-2.13.5.2).

2.13.5.1. Luminometer

A luminometer coupled to a low light video imager (Siemens) was used to detect colonies expressing green fluorescence. Long wave UV (366 nm) was used to excite the GFP-expressing colonies on the plate and the detection wavelength was set at 510 nm to coincide with the excitation and detection wavelengths for GFP. The agar plates with colonies were placed with their lids removed on the plate holder in the dark chamber. The UV light was turned on and the image was exposed at a photo UV setting that enabled collection of photons for 5 min. The image was then processed using a pseudocolor option that enabled easy analysis of GFP positive colonies.

2.13.5.2. Dissection microscope

GFP positive colonies were also visualized using a Leica UV Dissection microscope (Leica Wild MZ8) with excitation wavelength of 366 nm produced by a long wave UV lamp. Fluorescent and non-fluorescent colonies could be very clearly detected by microscopy.

2.14. Isolating PAH degrading bacteria from environmental samples

2.14.1. Isolation and enumeration of hydrocarbon degradative bacteria

This study was conducted with authentic PAH-contaminated soils that had undergone prolonged enrichment procedures in roller bottles (M. A. Pickard, personal communication). The degradative bacteria were recovered from soil samples (Table 2.4) by shaking ~10 g wet weight of soil in 90 ml of 0.1% sterile sodium pyrophosphate buffer (pH 7) containing glass beads for 1 h (Aislabie et al., 1997). The suspension was serially diluted and plated on appropriate PAH containing selection plates (Appendix B). The plates were incubated at 28°C for 7 days and colonies were purified for further study.

Isolation of degraders was also attempted by shaking ~ 3g of soil in 25ml phosphate buffer or mineral medium (in 125 ml flasks) overnight on a gyrotary shaker (200 rpm) and plating dilutions of the supernatant. To provide a C source for the MM agar, naphthalene crystals were placed on the lids of the petri dishes, which were then sealed with Parafilm^{TM} and incubated upside down. Non-volatile PAHs were dissolved in diethyl ether and provided by spray plating (Kiyohara et al., 1982) or by using an agarose overlay plate method (Bogardt and Hemmingsen 1992) as described in Appendix B.

2.14.2. Strain characterization

Gram staining was used as the first characterization step. A series of biochemical tests were then performed, including, oxidase, catalase and gelatin utilization tests (Appendix B). Substrate utilization was tested using API NE strips (Biomerieux, France), using manufacturer's instruction. Pigment analysis was carried out by a spectrophotometric method (Pollock, 1993) using a Philips PU8740 UV/VIS scanning spectrophotometer. Pigment producing strains were grown at 28°C for 2 days on a PAH selection plate. About 100 µl of cells were collected from the agar surface and placed in a 1.5 ml microcentrifuge tube. Methanol (0.5 ml) was added to extract methanol-soluble pigments at 50°C for 5 min. After centrifugation at 13,000 rpm for 5 min at 25°C, the supernatant was scanned (wavelength 200-400 nm) for absorbance using a methanol blank.

2.14.3. Plasmid isolations, curing, Southern blotting and hybridization studies on plasmid DNA from environmental isolates

Plasmids were isolated by a modification of the method of Kieser (Kieser, 1984) and Casse et al. (1979) as described in Sections 2.11.2 and 2.11.3. Vertical or horizontal agarose gels were run to visualise plasmid DNA (Section 2.11.6).

Two-dimensional gels were used to determine whether the isolates harbored multiple plasmids or different plasmid conformations (Mickel et al. 1977). Contour-

Table 2.4. Soil samples used for exogenous and endogenous plasmid isolations

Contamination type	**Origin of soil**	**Soil designation**
petroleum	Montreal, Que	MTL
creosote	Edmonton, AB	EDM
petroleum	Devon, AB	DEV
creosote	Prince Albert, SK	PAA

clamped Homogeneous Electric Field (CHEF) gel electrophoresis (Appenedix C) was also performed by C. Baraniecki on some of the isolates (*P. aeruginosa* PAA and *Pseudomonas* sp. Devon) to determine if they harbored large plasmids. Well-defined catabolic plasmids such as Bios2 (74 kb), NAH7 (83 kb), pWW0 (115 kb), LP6a (63 kb) were used as size markers.

Plasmid curing was attempted to determine if degradative genes were chromosomal or plasmid-encoded by repeated transfer in LB medium in 18 x 150 mm test tubes on a tube roller at 25°C. The transfer cultures were streaked first on LB, then onto MM + PAH plates after every transfer, screened for phenotype and tested for the presence of the plasmids.

For hybridization studies, plasmid DNA from the isolates was transferred to Hybond-N nylon membrane (Amersham Corp) by a modification of the method of Rigaud et al. (1987) as described in Section 2.11. The pGJZ 1707 probe (Goyal and Zylstra, 1996) bearing genes for phenanthrene dioxygenase and phenanthrene *cis*-dihydrodiol dehydrogenase (kindly donated by Dr. G. Zylstra, Rutgers, NJ) was labeled by random primer labeling by D. MacFarlane and used for hybridization studies (Section 2.11.2.).

2.14.4. Exogenous Isolation of plasmids

Exogenous plasmid isolations were carried out based on the procedures developed by Bale et al. (1987, 1988) and Top et al. (1994). Five grams of soil were shaken in a gyrotory shaker for 1 h in 45 ml of saline in 125 ml flasks with glass beads on the day of mating. The soil portion was allowed to settle for 15 min and the supernatant was used as donor for mating. Donors from the soil suspension were enumerated on MM + PAH plates. Two hundred microlitres of donor and recipient cells (*P. putida* KT2440Rif^r or *Pseudomonas* sp. Devon as recipient) were used, estimated by plate count to have a cell density of 10^9 cfu/ml. The donor and recipient cell suspensions were filtered onto a filter disc (Section 2.4.1) and incubated at 25°C for 24 h. Bacteria on the filters were then resuspended in 4 ml of 0.85% saline by vortexing vigorously for 2 min, serially diluted and plated on the MM + PAH plates with antibiotic selection. The PAHs used are listed in Section 2.2.

In an alternate method of mating, 0.5 ml each of donor suspension, recipient cells and LB were mixed in a 1.5 ml Eppendorf tubes, and incubated at room temperature for 24 h, after which samples were serially diluted and plated on MM PAH plates with antibiotic selection. For negative controls, soil suspensions and recipients were incubated separately under the parallel conditions and plated on the selection media. The fungal growth inhibitors cycloheximide (200 μg/ml) and benomyl (2 μg/ml) were added to all the plates to prevent growth of soil fungi. Replica plating was performed on colonies arising on selection plates and presumptive transconjugants were screened for plasmids as described earlier (Section 2.11). Positive controls were also performed as described above using *P. putida* NCIB 9816 (NAH7) as the self-transmissible plasmid and *E. coli* (R3006) as the non self-transmissible plasmid.

Statistical Analysis

A Tukeys HSD was performed to determine significant differences among treatments (Sprinthall, 1987).

Chapter 3

Results and Discussion

3.1. Conjugal transfer of TOL and NAH7 plasmids

This thesis is based on study of the conjugal transfer of two catabolic plasmids TOL and NAH7. In this thesis, pWW0 is referred to as "TOL", as it represents the isofunctional family of TOL plasmids and is commonly referred to as "TOL" in the literature. The 83 kb NAH7 plasmid is the archetype of the NAH plasmid family and is responsible for the degradation of naphthalene (Yen and Gunsalus, 1982) and this involves formation of salicylate and catechol as the intermediates.

Polycyclic aromatic hydrocarbons (PAHs) are widespread in nature. Microorganisms indigenous to contaminated sites often have a limited ability to degrade xenobiotic pollutants which are highly substituted or which have especially novel chemical structures (Pipke et al., 1992) and horizontal gene transfer might be a way for the native population to adapt to the selective pressure of such contaminants. Genetic exchange is a cooperative action within a population, allowing the adaptive information that has arisen in one microorganism to be disseminated through a population. Genetic exchange is especially important in preventing overspecialization within a microbial population (Atlas and Bartha, 1981).

Transformation as a mechanism of transfer of TOL and NAH7 plasmids was also explored in this thesis to ascertain that conjugation is the primary mechanism of plasmid transfer and discount the occurrence of transformation. Transformants were not observed on selection plates (MM + *m*-tol+ Rif or MM + Rif + naphthalene respectively) indicating that the results are due to conjugation.

Williams et al. (1996) demonstrated *in situ* transformation of the plasmid pQM17 (7.8kb in size) that encodes mercury resistance into *Acinetobacter calcoaceticus*. Studies on natural transformations have used small plasmids. Probably uptake of large plasmids like TOL or NAH7 by transformation does not occur or the conditions provided in the experiment might not be conducive for transforming the recipient. In nature, where free DNA would be exposed to restriction endonucleases and competent recipients might not be present at high cell densities, transformation might not be a viable mechanism for the uptake of large plasmids.

There is still a lot to be understood about the physiology, ecology and genetics of soil microbes and it is impossible to test the multitude of interactions that exist in nature. Transfer of degradative plasmids might result in genetic rearrangements which would result in microevolution. Studying gene dissemination under different environmental conditions will help us understand the dynamics and flux involved during transfer of such large catabolic plasmids.

3.1.1. Search for suitable conjugal recipients

Several *Pseudomonas* strains were tested for suitability as conjugal recipients of the TOL plasmid pWW0. *P. putida* mt-2 (TOL) was used as the donor in plate matings with *P. aeruginosa* AK1401, *Pseudomonas* sp. Devon (isolated from Devon soils; Appendix A) or *P. alcaligenes* DM201 as recipients. The first two species gave rise to putative transconjugants at a very low frequency ($<10^{-8}$ transconjugants/recipient), while the latter species yielded no transconjugants in three trials. Selection was achieved using *m*-toluate and the appropriate antibiotic; parental donor and recipient control plates did not show any growth on selective media. Plasmid preparations from several randomly selected presumptive transconjugant colonies confirmed the acquisition of the TOL plasmid (data not shown). Parallel plate matings were carried out with *P. putida* PpG7 (NAH7) as the donor and the above mentioned recipients, as well as rifampicin resistant mutants of *P. putida* MFY (an environmental isolate from Montreal soil) and *P. putida* NCIB10643. *P. putida* KT2440 was the ideal recipient for the transfer of these large catabolic plasmids, probably owing to its res^-mod^- phenotype. The other recipients' restriction systems may have prevented maintenance or establishment of the NAH7 plasmid, or lack of expression of the catabolic function in the new host may have prevented its detection, or unsuitable conjugation conditions may have been used. This experiment showed that interspecific transfer of the TOL and NAH7 plasmids is possible and expression of the catabolic phenotype also takes place in the new host.

Christensen et al. (1998) studied TOL plasmid transfer into three different recipients and reported that *P. putida* KT2442 was the most suitable recipient. Similar results were observed in our study with the related recipient KT2440. This might be due to the lack of restriction modification systems in the recipient (Mamool et al., 1986), enabling the successful establishment of the plasmid in the recipient.

It is not surprising that most studies on horizontal transfer of catabolic plasmids have used *P. putida* KT2440 as the recipient (Christensen et al., 1996). Bale et al. (1988) reported that in their host range studies with pQM1, a mercury resistance plasmid, conjugation is limited to fluorescent pseudomonads and that little or no transfer occurred between more distantly related species.

These current studies and the previous work of Christensen et al. (1998) have shown that conjugal transfer and expression of large catabolic plasmids takes place, although at very low frequencies, under laboratory conditions. The frequency of gene transfer in the soil is limited by a number of biological and physicochemical characters and it is important to recognize the impact of these factors on conjugal transfer. Therefore, it would be ideal to study conjugal transfer of TOL and NAH7 to organisms isolated from natural environments.

3.2. Mating studies on agar surfaces

3.2.1. Mating studies with *P. putida* mt-2 (TOL)

Conjugal transfer of bacterial plasmids has been demonstrated on solid surfaces and in broth. TOL is the best studied of the transmissible degradative *Pseudomonas* plasmids and belongs to the Inc P9 group (Williams and Murray, 1974). The objective of this experiment was to detect TOL plasmid transfer and establish the conjugation frequency on plates on agar surfaces in comparison with broth mating.

Three methods were used to mate *P. putida* mt-2 (TOL) with *P. putida* KT2440Rif^r: direct mating on an agar surface, filtering onto a nitrocellulose membrane incubated on an agar surface, and mating in a stationary broth culture. When the mating mixtures were plated onto selective agar to determine conjugation frequency, typically the presumptive transconjugants formed too many colonies at the 10^{-2} dilution to count accurately and no colonies at the next highest decimal dilution, regardless of the diluent used (LB or phosphate buffer). However, accurate estimation of conjugation frequency was made possible by carrying out a two-fold dilutions rather than decimal dilutions when such problems were encountered. Recipient control plates did not show any colony growth.

The three methods tested gave results in the same order of magnitude (Table 3.1). One of the likely reasons for such an observation could be because of transfer of residual LB medium from the plates or the broth. This might enable growth of the transconjugants on the selection plates by providing a readily available source of carbon. All the presumptive transconjugants tested grew on *m*-toluate and harbored the TOL plasmid.

Plasmid preparations were performed on the randomly selected presumptive transconjugants [*P. putida* KT2440Rif^r (TOL)], called the KTT series. Some (20%) of the presumptive transconjugants (<100 transconjugants/mating) harbored plasmids which were smaller (86 kb) than the wild type TOL plasmid (115 kb) (Figure 3.1). These plasmids were considered to be TOL plasmids which had an amount of DNA deleted from them and were called ΔTOL, or "TOL deletion plasmid", and are discussed in Section 3.3. The transconjugants with the ΔTOL plasmids could still grow on *m*-toluate thereby, indicating that the catabolic region had not been deleted, and indeed the method of selection (growth on *m*-toluate) required that the catabolic region be retained.

In this experiment it was not possible to determine if TOL plasmid transfer was favored by a surface conjugation system, as transfer frequencies were almost identical when mating was performed in broth as well as on the surface of agar plates. In the case of the TOL plasmid, this experiment would indicate that conjugal transfer is an effective method of gene transfer and both solid surface and liquid favour conjugal transfer. This

Table 3.1. Frequency of conjugation of TOL plasmid pWW0 to recipient *P. putida* KT2440Rif^r by different mating techniques

Mating technique	Conjugation frequency (# of transconjugants/recipient) [c]
Filter mating[a]	1.3 ± 0.9 x 10^{-7}
Plate mating[a]	2.0 ± 0.6 x 10^{-7}
Broth mating[b]	1.0 ± 0.6 x 10^{-7}

[a] all matings were performed on LB plates without selection pressure. Approximately 10^9 cells/ml of donors and recipients were used for mating

[b] mating performed in stationary LB broth

[c] conjugation frequencies are the means of triplicates ± standard deviation.

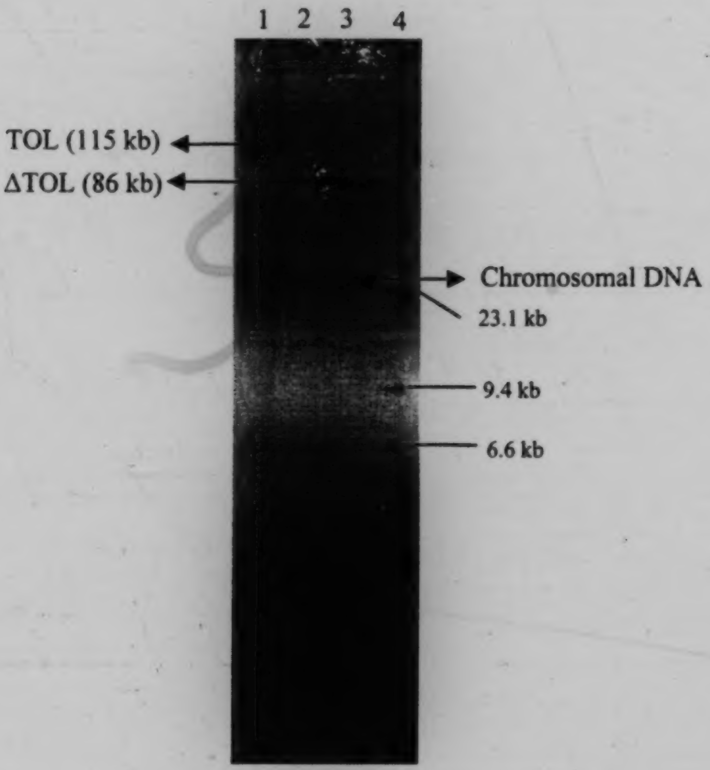

1
2
3
4
TOL (115 kb)
ΔTOL (86 kb)
Chromosomal DNA
23.1 kb
9.4 kb
6.6 kb

Figure 3.1. Undigested plasmid DNA from strains harboring TOL (wild type) and deletion TOL (ΔTOL). Lanes: 1. TOL wild type; 2, 3. ΔTOL; 4. λ*Hind*III

could be especially important when organisms with TOL are used for bioaugmentation in aqueous and terrestrial habitats. Stratum preferences give clues as to the nature of the habitat in which conjugal transfer of plasmids occur.

TOL is reported to encode a 'surface-preferred' conjugation system (Bradley, 1980) as opposed to a 'surface obligatory or a 'universal system'. Bradley and Williams (1982) showed that TOL plasmid pWW0 transferred 17-fold better on a surface than in liquid. They also reported that wild type TOL is naturally de-repressed for transfer in its original host *P. putida* mt-2 and that it determines thick flexible conjugative pili constitutively.

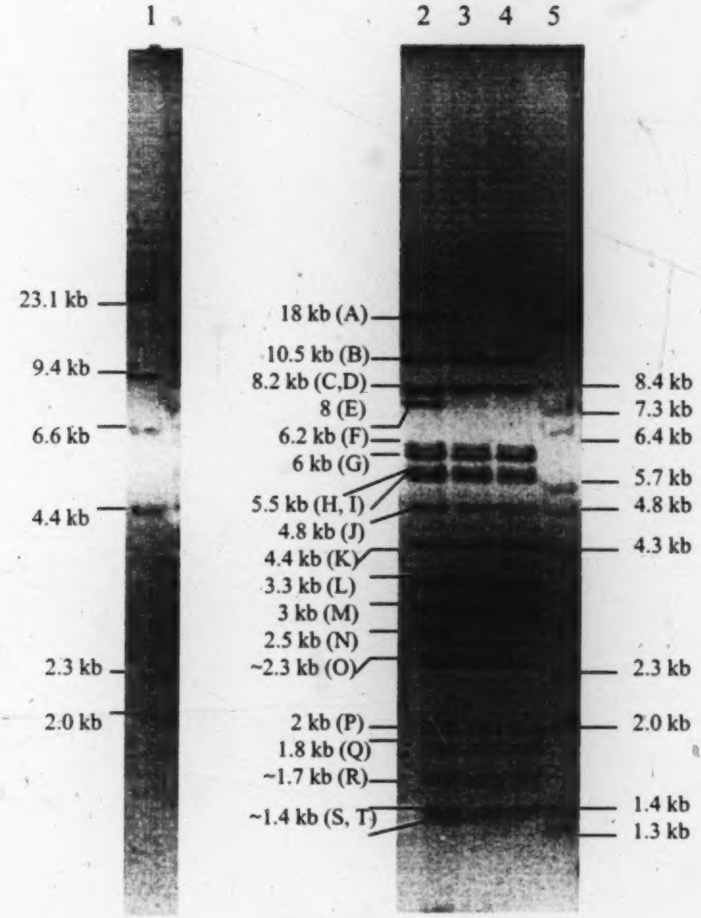
TOL plasmid transferred at a detectable frequency (ca. 10^{-7} transconjugants/recipient) into *P. putida* KT2440 which is plasmid free and there were no stratum preferences for conjugal transfer. Perhaps, a factor that could have influenced this observation of equivalent frequency of transfer on solid surface and liquid in our experiment could be the sensitivity of detection of transconjugants harboring TOL on the selection plates. Transfer of TOL plasmid was equally efficient in solid as well as on liquid surfaces. Although the *tra* genes and conjugal transfer mechanism of TOL plasmid has not been well studied, it might be comparable to the transfer mechanism of RP4, an IncP group plasmid (Section 1.2.3).

3. 3. TOL plasmid deletions

3.3.1. Spontaneous deletions of the TOL plasmid during horizontal transfer

When transferred to *P. putida* KT2440, the TOL plasmid was frequently subjected to a deletion event resulting in a ΔTOL plasmid. Plasmid sizes were determined by measuring the relative mobilities of the restriction fragments in 1.2% agarose gels. The Δ TOL plasmids, pKTT4 and pKTT13, are 86 kb (Figure 3.1) in size and are characterized by the loss of 18 kb, 8 kb and 2.5 kb *Eco*RI fragments compared with wild type TOL (Figure 3.2).

The catabolic genes of the TOL plasmid pWW0 are organized into two operons, referred to as the upper and lower pathway operons. The upper pathway operon, *xylCAB*, encodes the degradation of toluenes and xylenes to benzoates and toluates (Harayama et al, 1986). For detailed description of pathways and genes involved, refer to Figures 1.1 and 1.2 and Table 1.1. The missing 18 kb *Eco*R1 fragment A corresponds to the *xylAB* genes which encode xylene oxygenase and benzyl alcohol dehydrogenase, as seen from Figure 3.3. However, the deletion mutants could grow on *m*-toluate as well as toluene and xylene. The *xylC* region might not have been lost. Internal transposition and recombination could have eliminated a part of the cryptic region and the *xylAB* genes could be in an alternative location on the chromosome of the recipient. It might be interesting to probe the ΔTOL plasmid and the chromosome of the transconjugant to determine the presence of the *xylAB* genes. Another unlikely explanation for the deletion pattern could be that the *P. putida* KT2440 recipient itself might have xylene oxygenase and benzyl alcohol dehydrogenase functions. The 8 kb *Eco*R1 fragment E might correspond to the lower pathway genes or alternatively if the doublet is not considered, it might be *Eco*R1 fragment D corresponding to *xylS* and *xylR*. Substrates for the upper pathway enzymes such as toluene or *m*-methyl benzyl alcohol are activators of the pathway when *xylR* is present. Similarly *m*-toluate is both a substrate and an inducer for the lower pathway in conjunction with the *xylS* gene product (Burlage et al., 1989).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2 3 4 5
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6.6 kb
4.4 kb
2.3 kb
2.0 kb
18 kb (A)
10.5 kb (B)
8.2 kb (C,D)
8 (E)
6.2 kb (F)
6 kb (G)
5.5 kb (H, I)
4.8 kb (J)
4.4 kb (K)
3.3 kb (L)
3 kb (M)
2.5 kb (N)
~2.3 kb (O)
2 kb (P)
1.8 kb (Q)
~1.7 kb (R)
~1.4 kb (S, T)
8.4 kb
7.3 kb
6.4 kb
5.7 kb
4.8 kb
4.3 kb
2.3 kb
2.0 kb
1.4 kb
1.3 kb

Figure 3.2. *Eco*RI digested TOL plasmid and its deletion derivative ΔTOL Lanes: 1, λ*Hind*III; 2, wild type pWW0; ΔTOL plasmids: 3,pKTT4 and 4, pKTT13;5, λ*Bst*EII

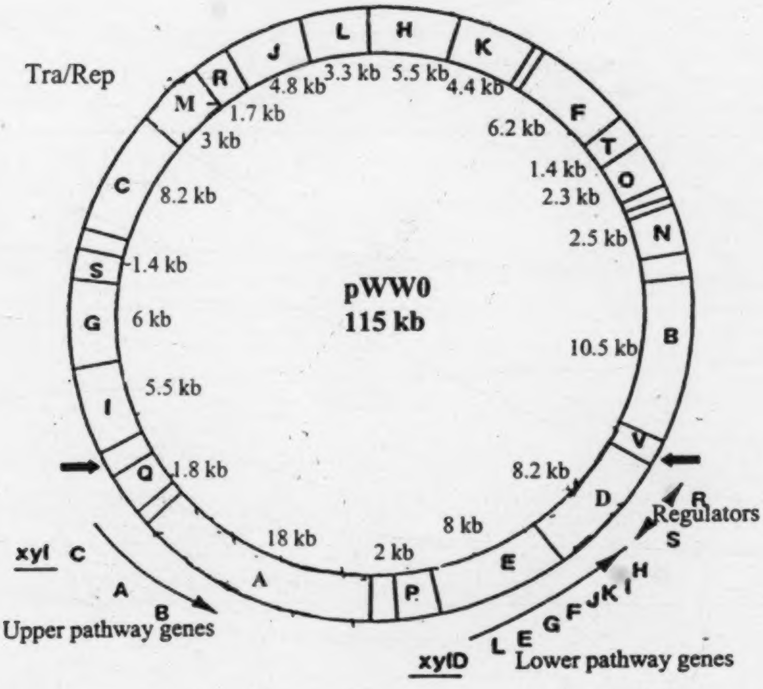

Tra/Rep
R
J
L
H
K
M
4.8 kb
3.3 kb
5.5 kb
4.4 kb
1.7 kb
3 kb
F
6.2 kb
T
1.4 kb
O
2.3 kb
C
8.2 kb
2.5 kb
N
S
1.4 kb
pWW0
115 kb
G
6 kb
B
10.5 kb
5.5 kb
I
V
Q
1.8 kb
8.2 kb
D
R
Regulators
S
8 kb
18 kb
2 kb
xyl C
A
E
A
P
H
B
I
K
J
F
G
Upper pathway genes
E
L
xylD
Lower pathway genes

Figure 3.3. Physical map of TOL plasmid pWW0 with *Eco*R1 restriction sites and known genes (digestion fragments labeled A-T). Block arrows indicate 1.4 kb inverted repeats. Adapted from Lehrbach et al. (1982); Harayama and Don (1985); Tsuda and Iino(1987).

For gene regulation in pWW0, refer Figure 1.3. It seems to be interesting in this study that the entire upper and lower pathway could in fact be functional in the deletion mutants without the degradative pathways and the regulators. In the ΔTOL plasmids observed in this study, the regulation could be mediated by a chromosomal regulator in *P. putida* KT2440. An interesting observation in the ΔTOL mutants is that their hosts are not deficient in degradative functions but only in transferability (detailed discussion follows in section 3.3.2). Therefore, the 2.5 kb *Eco*R1 N fragment missing in ΔTOL might be encoding part of the transfer and replication (*tra rep*) functions of the plasmid and this might bestow lesser conjugative ability on the ΔTOL plasmid, although this seems improbable. However, it also seems improbable that three different deletion events could occur in the TOL plasmid rather than deletions of contiguous areas. Alternatively, it can be proposed that *Eco*R1 fragments A, P and E corresponding to *xylAB*, and *xylDLEGFJ* could have been lost. But this might not account for the fact that *P. putida* harboring ΔTOL could still grow on the provided substrates. Another strong contender could be the loss of *Eco*R1 fragments A, Q and I and this might reconcile with the lowered transfer function of the ΔTOL plasmid (refer Section 3.3.2). It should also be recognized that these predictions are based on the available digests and taking into account the doublets, and strong conclusions might not be possible with the limited data. It is difficult to estimate the accurate sizes of the digested fragments and doublets are difficult to resolve. These predictions are also based on the available restriction map (Tsuda and Iino, 1987) and the veracity of the map itself could be doubtful. Another probable explanantion could be that our plasmid might have diverged from the original TOL plasmid of Worsey and Williams (1974).

Deletions are common in the TOL plasmid. The deletion mutants observed in the current study still retained the *m*-tol$^+$ phenotype, as they were selected on *m*-toluate and there was no "benzoate curing" involved.

Bayley *et al.* (1977) reported a spontaneous deletion of a specific region of about ca. 42 kb from the TOL plasmid when selected on benzoate. Since the deletion plasmid lost the Tol$^+$ phenotype, the presence of "hotspots" was suggested for the excision of a segment that codes for at least part of the TOL pathway. Pickup and Williams (1982) reported that, during growth on benzoate, TOL plasmid pWW20, a 270-280 kb plasmid, underwent extensive deletions of 90-100 kb and the host strains lost their ability to grow on *m*-toluate. "Benzoate curing" resulting in deletions in pWW0 and related plasmids might result from benzoate directly invoking TOL excisions by transposase activation, or favoring selection of preexisting spontaneous deletion mutants through increased fitness of the deletion mutant (Leddy et al., 1995). Similarly, Don and Pemberton (1985) reported a 40 kb deletion of the 2,4-dichlorophenoxyacetic acid (2,4-D) degradative plasmid pJP4 after selection on 2,4-D for the loss of catabolic functions.

Williams et al. (1988) proposed that benzoate curing or deletion in pWW0 in *P. putida* occurred spontaneously and that deletion mutants out-competed wild type cells because of a growth advantage on benzoate. Jeenes and Williams (1982) reported that the plasmid harbored by *P. putida* WR211, a transconjugant resulting from the acquisition of the pWW0 degradative plasmid, had suffered a deletion of 39 kb from the catabolic region. They showed that WR211 contained the missing 39 kb, together with at least an additional 17 kb of pWW0 DNA, integrated in the chromosome. Unstable maintenance of the pWW0 derivative plasmids in *E. coli* allowed the identification of two transposable elements, Tn*4651* (56 kb) and Tn*4653* (70 kb) that completely encompasses Tn*4651* (Tsuda and Iino, 1987). The catabolic genes on pWW0 are located within the transposons, Tn*4651* and Tn*4653*, and the transposition of each element actually contributes to the dissemination of the toluene degrading genes on various replicons in some *Pseudomonas* strains (Tsuda and Iino, 1988).

Selective loss of a catabolic pathway is not necessarily lethal provided that an alternate carbon source is available to sustain viability and growth; the pathway could be restored under more favorable conditions via genetic exchange (Leddy et al., 1995). Leddy et al. (1995) hypothesised that irreversible loss of catabolic functions, though seemingly counterproductive, might increase organism fitness or survivability. They showed that deletions or excisions occurred within the toluene degradative pathways in strain *P. putida* 54g and these mutants failed to revert to tol$^+$.

Potential instability, as manifested by deletions, was seen in the TOL plasmid at a frequency of 20% when transferred into *P. putida* KT2440. One of the questions that needs to be answered is whether the deletions are due to the new host, i.e. some property of the heterologous host, or a result of the transfer process itself. The TOL plasmid in its wild type host (*P. putida* mt-2) did not exhibit such deletions even under non-selective conditions. This event might be due to some unknown transposase functions in the new recipient. In spite of *P. putida* KT2440 being a laboratory-adapted strain and also well-studied, transposase functions have not been demonstrated to exist chromosomally on this organism. Ramos-Gonzalez et al. (1994) reported that the TOL plasmid pWW0 is able to mediate chromosomal recombination in the canonical unidirectional way and also bidirectionally i.e. by regular conjugal transfer and also retrotransfer. They hypothesised that the supercoiled structure of different regions in the recipient chromosome might favor the transposition event and the mobilization of genes (Km^r marker) via cointegration with the plasmid, or that the TOL plasmid might integrate into the recipient chromosome by site-specific recombination.

Superficially different TOL plasmids have been isolated from natural habitats: deletions, duplications and subsequent recombinations might be the reason for such diversity. O'Donnell and Williams (1991) reported duplication of both upper and lower *xyl* catabolic operons on the 250 kb TOL plasmid pWW15 during routine maintenance of the strain. They reported a duplicated upper pathway operon that remained stable under most growth conditions. In contrast, spontaneous deletions of pWW0 were not observed in this study even when the host was maintained on non-selective carbon sources such as succinate, acetate or nutrient broth.

Comparative studies with various toluene and naphthalene-degrading plasmids suggest that the present organization of the genes for the complete degradative pathway for each compound must have been established by physical assembly of a common region comprising the *meta*-pathway gene cluster together with other appropriate blocks of catabolic genes by undefined mechanisms (Cane and Williams, 1986; Shaw and Williams, 1988; Tsuda and Iino, 1990). This might be the reason for the isolation of toluene and naphthalene degradative plasmids of different sizes from the environment and such deletions and duplications might be commonplace in nature.

3.3.2. Effect of TOL plasmid deletions and plasmid hosts on conjugal transfer

TOL plasmid transfer was studied in sterile sand microcosms. We tried to determine if the deletion event on the TOL plasmid significantly altered the transfer functions and also the influence of the host on TOL plasmid transfer to a recipient. Donors used included *P. putida* strains ATCC33015 (TOL), KT2440 (TOL) and KT2440 (ΔTOL). The recipient was a plasmid free, *m*-tol$^-$ nalidixic acid resistant strain of KT2440. Selection pressure was provide as *m*-toluate (see Section 2.8.2).

It was observed that the wild type TOL plasmid transferred at a high frequency only when it was present in its natural host *P. putida* ATCC33015 (Figure 3.4). Transfer was less efficient when KT2440 (TOL) was used as a donor. The ΔTOL plasmid transferred at a very low frequency (lower detection limit ca. 10 transconjugants). This could be due to the transfer function of the TOL plasmid being affected as a result of the deletion. Restriction digestion analysis revealed the loss of the 2.5 kb *Eco*RI fragment N which might correspond to the region responsible for transfer functions and replication (Section 3.3.1). The apparently improved frequency after 48 h could be due to vertical transfer as well as waves of horizontal transfer (see Section 3.7.2).

The TOL plasmid is naturally derepressed for transfer in its natural host (Bradley and Williams, 1982). This might account for the higher transfer frequency observed when TOL is present in *P. putida* ATCC33015. Constitutive expression of the tra$^+$ genes on the TOL plasmid pWW0 accounts for the large number of pili on the surface of *P. putida* (Bradley and Williams, 1982).

Horizontal plasmid transfer alone would not help in the successful establishment of degraders in a contaminated environment. Christensen et al. (1998) suggested that the

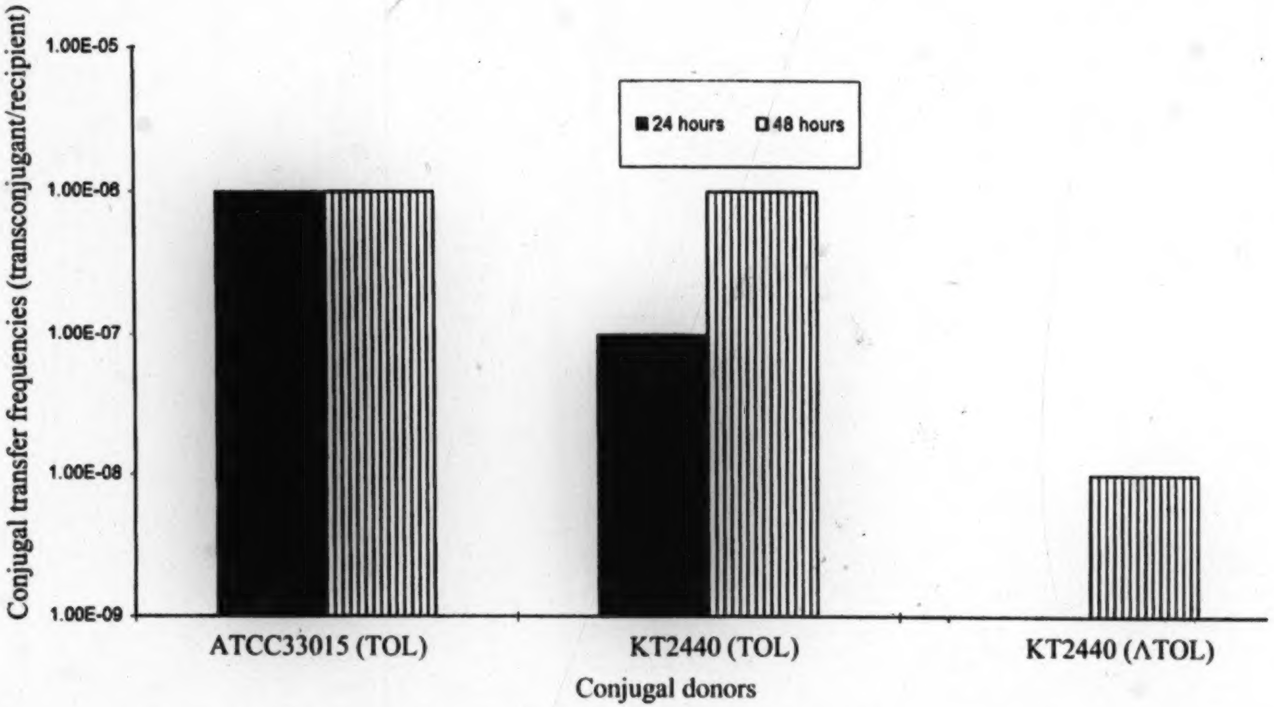


Figure 3.4: Conjugal transfer of TOL or ΛTOL from *P. putida* strains ATCC33015 (TOL), KT2440 (TOL) and KT2440 (ΛTOL) to *P. putida* KT2440 m-tol$^-$Nah$^-$, determined after 24 h or 48 h mating contact.

dominant mode of establishment of the TOL plasmid in the community they studied was through the rapid growth of cells, i.e. vertical plasmid transfer representing an optimal host-plasmid combination. Actual plasmid transfer played a quantitatively minor role, which only became significant in situations where the donor strain could not establish itself directly.

3.4. Mating studies with *P. putida* PpG7 (NAH7)

3.4.1. Spontaneous insertions on the NAH7 plasmid

Conjugal transfer of the NAH7 plasmid from *P. putida* strain PpG7 to KT2440Rif^r produced transconjugants selected for their ability to grow on naphthalene. However, transfer resulted in apparently stable insertion events in the plasmid (~4 insertion events for every 18 colonies screened for plasmids). The transconjugants, called KTN-series, harbored different types of plasmid profiles and the insertion event produced 2 or 3 plasmids in some transconjugants (insertion or +NAH7 plasmids). Restriction digestion analysis was then carried out on the KTN-series of mutant plasmids and compared to the wild type NAH7 plasmid (Figure 3.5).

Insertion plasmid pKTN2 was ca. 93 kb in size, having acquired an additional 10 kb *Eco*RI fragment compared with the wild type NAH7 plasmid. It was difficult to establish the presence of any additional low molecular weight *Eco*R1 fragments due to problems of resolution on agarose gel. Another high molecular weight plasmid, pKTNF2, differed from the NAH7 wild type in that it had extra 14 kb, 10.5 kb and 4.9 kb *Eco*RI fragments (figure not shown), but it was unstable and was lost during repeated cultures of

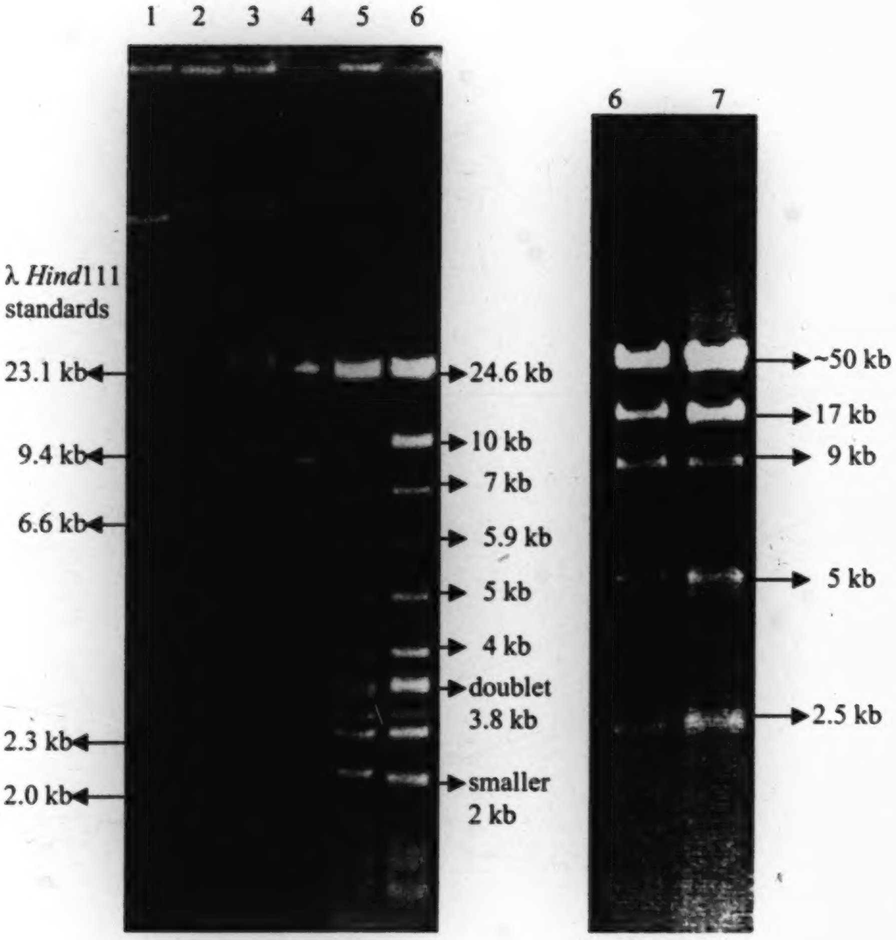

1 2 3 4 5 6
6 7
λ *Hind*111 standards
23.1 kb
9.4 kb
6.6 kb
2.3 kb
2.0 kb
24.6 kb
10 kb
7 kb
5.9 kb
5 kb
4 kb
doublet 3.8 kb
smaller 2 kb
~50 kb
17 kb
9 kb
5 kb
2.5 kb

Figure 3.5. Undigested, *Eco*RI-digested and *Sma*l digested plasmid DNA isolated from wild type *P. putida* PpG7 (NAH7) and *P. putida* KT2440Rif^r transconjugants harboring +NAH7 plasmids

Lanes: 1, 2, 3, undigested plasmids: 1, wild type NAH7; 2, +NAH7-pKTN2 in transconjugant KTN2; 3, +NAH7-pKTN6 in transconjugant KTN6; 4, λ*Hind*III; 5, 6, *Eco*RI-digested plasmids: 5, wild type NAH7; 6, +NAH7 (pKTN2); 7, 8, *Sma*l-digested plasmids (Appended figure): 7, wild type NAH7; 8, +NAH7-pKTN2.

the transconjugant. A *Sma*1 digest is also included in Figure 3.5 as literature reports include digestion maps with *Sma*1 and only a partial *Eco*R1 map is available for comparison (Yen and Serdar, 1988). However, the *Sma*1 digest is not informative in the current study as it yielded similar banding patterns with wild type NAH7 and insertion NAH7 plasmids, probably because the largest *Sma*1 fragment is ~50 kb in size and lies beyond the largest size exclusion limit of agarose gel electrophoresis.

In the current study gene duplications of NAH7 in the transconjugants were observed even without environmental stress. Stability of the insertions was assessed by repeated culture of the transconjugant without nutritional selection. Even after 18 transfers in rich medium, the insertions were maintained as a single plasmid species.

This plasticity of the plasmid suggests the presence of transposon-like activities. Tsuda and Iino (1990) demonstrated that the *nah* genes (32.5 kb) on NAH7 are carried on a defective transposon, Tn*4655*, which is mobilized by the Tn*4653* transposase. In this study, the observed insertions could have arisen by transposition and recombination events in the new host, probably due to the activation of the transposase function or complementation by a chromosomal transposase. In the current study, the wild type NAH7 plasmid might have diverged in our laboratory accounting for the discrepancy in digestion patterns compared to the wild type (refer Figures 3.5 and 3.7).

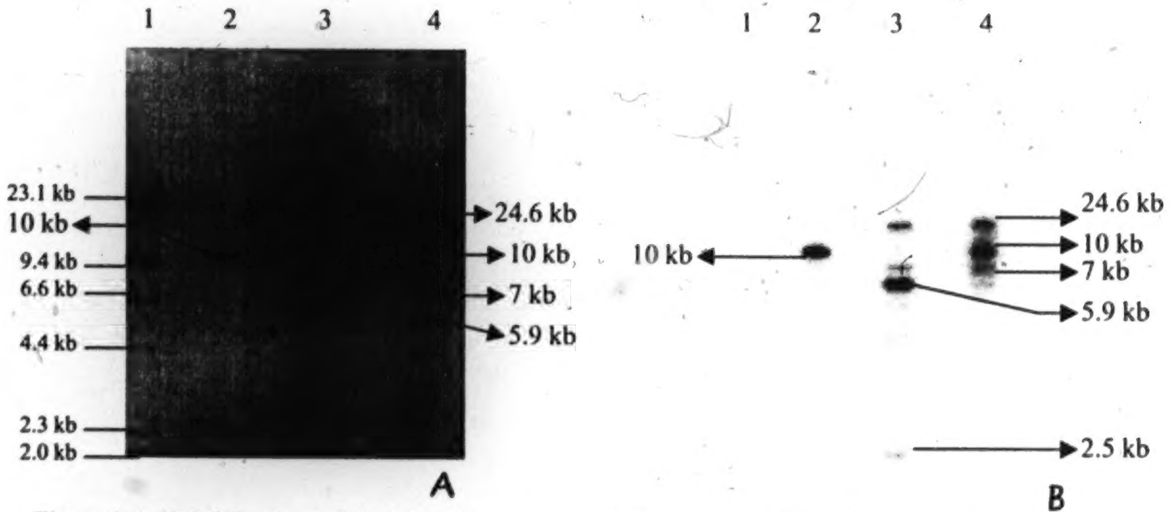
O'Donnell and Williams (1991) suggested certain selective advantages which gene duplications might confer to isolates in the environment. Heteromultimeric enzymes with advantageous properties might be formed between the gene products of the duplicate copies, or the regulation of the duplicated genes might result in their being expressed under different environmental conditions so that if the conditions fluctuate that there is intermittent selection for maintenance of both copies. They also suggested that gene conversions occurred between the duplicate copies to ensure a high level of repair of mutations.

Gene duplications *per se* are not stable (O'Donnell and Williams, 1991). Tandem duplications of catabolic genes have been induced under selective pressure using some of the deletion derivatives of pWW15, but these mutants were highly unstable under non-selective conditions and rapidly lost the multiple gene copies (Keil and Williams, 1985). Ghosal and You (1988) reported that the 2,4-D degradative plasmid pJP4 contained an inverted duplication of 24.5 kb when grown under 3-chlorobenzoate selection. They also suggested that gene duplication and subsequent mutation of the duplicate copy of the catabolic genes is a likely mechanism for broadening the range of growth substrates for *Alcaligenes* sp. harboring the degradative plasmids. Efficient recombination functions present in all biological systems are the most important challenge to the maintenance of large duplications, especially in the absence of selective pressure. Ghosal and You (1988) showed that tandem duplications are more stable than inverted duplications when present in natural populations and that they might be tolerated for many generations in exponentially growing wild-type hosts and could later be enriched under appropriate selective pressure. O'Donnell and Williams (1991) questioned the existence and origin of duplications: they stated that they resulted either from tandem duplications within a plasmid and subsequent mutational divergence, or from a recombination event occurring between two different catabolic plasmids co-existing within the same cell.

It is interesting to note in our experiment that the insertions on NAH7 were very stable even under non-selective conditions. The insertion mutation might confer an advantage on the recipient, enabling better growth on naphthalene. Alternatively, the existence of the insertion mutant and the wild type plasmid might be merely transitory in nature. In this light, the maintenance of the wild type NAH7 and + NAH7 together is intriguing. Why would both plasmids be maintained and why have recombinations not taken place between them? The two different plasmids could be contributing to a gene dosage effect. Duplications and deletions might be common mechanisms existing in nature solely to perform an evolutionary function. The archetypal TOL and NAH7 plasmids with their single copies of catabolic operons might not exist in nature. There might be an element of flux existing in nature enabling maintenance of multiple copies and excision of certain genes.

3.4.2. Identification of the insertion in NAH7

The +NAH7 plasmid was digested with *Eco*R1 and the inserted fragment (~10 kb) was purified, ^{32}P-labeled and used as a probe against the wild type NAH7 plasmid. Based on the hybridization pattern (Figure 3.6), and by comparison with the physical map of NAH7, (Figure 3.7), it was possible to identify the location of the insertion. The ~10 kb fragment hybridized strongly to the ~25 kb *Eco*R1 fragment A and the 5.9 kb fragment C on the NAH7 wild type plasmid that contain the *nah* operons (Yen and Serdar, 1988) and likely overlaps these two fragments (See Figure 1.4 and Table 1.2 for description of the napthalene oxidation pathway and genes involved). Weak hybridization was seen with a 7 kb and a 2.5 kb *Eco*R1 fragment. The insertion observed could have resulted from transposition of the entire cassette followed by intraplasmidic or plasmid-chromosome recombination events, after transfer in the new host.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7 kb
5.9 kb
A
1
2
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4
10 kb
24.6 kb
10 kb
7 kb
5.9 kb
2.5 kb
B

Figure 3.6. Hybridization of NAH7 and its insertion derivative +NAH7 with the 10 kb duplicated fragment from +NAH7 as the probe.
Panel A. Agarose gel
Panel B. Corresponding autoradiograph depicting hybridization of +NAH7 insertion fragment
Lanes: 1, λ*Hind*III; 2, purified 10 kb fragment from +NAH7 (positive control); 3, *Eco*RI digested NAH7; 4, *Eco*RI digested +NAH7

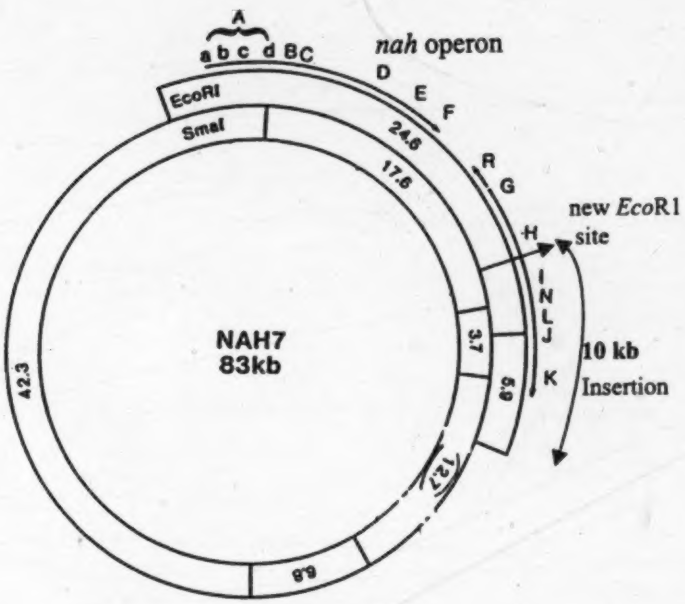
A
a b c d BC
nah operon
D
E
F
EcoRI
SmaI
24.6
17.6
R
G
H
new *Eco*R1 site
I
N
L
J
K
10 kb Insertion
3.7
5.9
12.7
6.8
42.3
NAH7
83kb

Figure 3.7. Physical map of NAH7 plasmid showing *Sma*1 and partial *Eco*R1 restriction sites and presumed insertion. The bold letters indicate the naphthalene degradative genes. Adapted from Yen and Serdar (1988).

It is interesting to note that spontaneous duplications were not seen when the NAH7 plasmid was maintained in its original host *P. putida* PpG7. The insertions were seen only on transfer into *P. putida* KT2440 and the event was observed with and without selective pressure (see Section 3.9.1). These insertions might be triggered by transfer to a new host and NAH7 plasmid transfer into other suitable hosts should be attempted to test the 'host hypothesis' in the duplication event.

3.4.3. Effect of insertion on transfer of the +NAH7 plasmid

It was surprising to find two plasmids of the same incompatibility group being retained in the same organism. A number of questions remained to be answered at this point. This experiment set out to determine whether the presence of two plasmids in the transconjugant affected transfer and whether the existence of the two plasmids, i.e. NAH7 and +NAH7 was merely transitory in nature, as was the high molecular weight pKTNF2 plasmid (Section 3.4.1).

It was found that pKTN2 transferred as efficiently to *P. putida* SM1396 as the wild type NAH7 and the presence of two plasmids in the same host did not interfere with the transfer functions. An interesting event that occurred was the segregation of the two plasmids (at a frequency of 4/18 transconjugants screened) in the new transconjugants obtained, resulting in isolation of strains F4 (NAH7) and F7 (+NAH7) as seen in Figure 3.8 a.

We attempted to determine if the insertion event had an effect on the transferability of the plasmid, i.e. to determine if F4 (NAH7) and F7 (+NAH7) transferred their plasmids at similar frequencies (as outlined in Figure 3.8 b). The recipient used was *P. putida* strain KT2440 Tn*5*-Km^r mutant and a 1:1 donor to recipient ratio used for plate mating gave a conjugation frequency of 10^{-6} transconjugants/recipient with both the donors. All transconjugants grew with naphthalene as the sole carbon source. This experiment showed that the higher molecular weight (+NAH7) plasmid had not lost any of its catabolic or transfer functions due to duplication or disruption of transfer functions and it was transferred as efficiently as the wild type NAH7 plasmid. Plasmid preparations performed on 96 selected transconjugants indicated that the F7 (+NAH7) plasmid was maintained in the transconjugants without apparent change, but the F4 (NAH7) plasmid, when transferred into the new host, underwent two duplication events which had resulted in the presence of two higher molecular weight plasmids other than NAH7 (figure not shown). The duplicated molecules were not comparable in size to the wild type NAH7 plasmid. This confirms the observation that the wild type NAH7 plasmid undergoes spontaneous duplication events as it is transferred into a new host. This event occurred at a frequency of 4/18 transconjugants screened. The insertion NAH7 plasmid as seen in F7 (+NAH7) was very stable and did not revert to the wild type or undergo any further change or resolve even after repeated transfers in non-selective medium. It remains a possibility that the insertion event observed during the transfer of the wild type NAH7 plasmid into a KT2440 recipient could be due to homologous recombination events or due to excision of the defective transposon present in NAH7 in the presence of the transposase which might be present in the new host and this event might occur only in *P. putida* KT2440 strain. A speculation for the maintenance of both NAH7 and +NAH7 in the same host could be due to an increase in the copy number. If

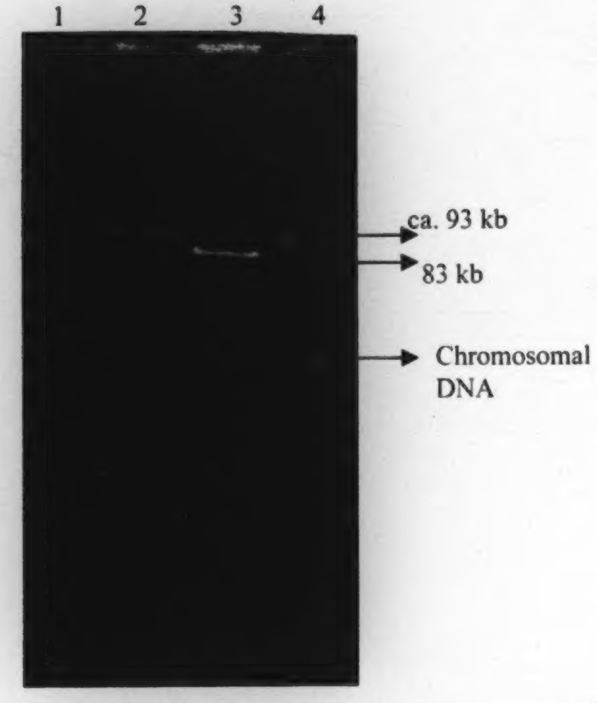
1
2
3
4
ca. 93 kb
83 kb
Chromosomal DNA

Figure 3.8 a. Agarose gel showing undigested +NAH7 and NAH7 plasmids Lanes 1. Wild type NAH7 (83 kb); 2, NAH7 and +NAH7 (93 kb) isolated from KTN2; 3, NAH7 isolated from *P. putida* F4 ; 4. +NAH7 isolated from F7

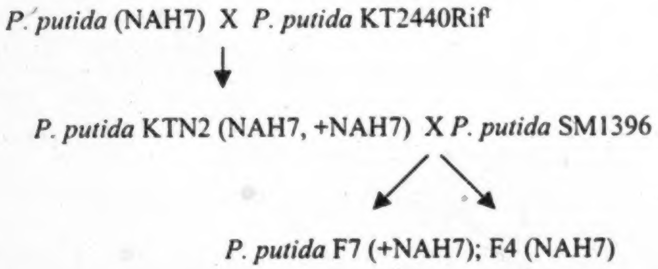
P. putida (NAH7) X *P. putida* KT2440Rif^r
P. putida KTN2 (NAH7, +NAH7) X *P. putida* SM1396
P. putida F7 (+NAH7); F4 (NAH7)

Figure 3.8.b.Schematic diagram showing parental strains, transconjugants and plasmids observed.

so, then the increased metabolic burden of having the second plasmid must be offset in some manner by the metabolic activation of having an extra (albeit larger) copy.

3.5. Influence of parental cell densities on conjugal transfer of NAH7

The objective of this study was to quantify conjugal transfer of the NAH7 plasmid from *P. putida* PpG7 (NAH7) to *P. putida* KT2440Rif^r on agar surfaces and to determine the effect of initial parental cell densities on mating frequency.

LB plates were used for mating. The standard filter mating procedure was adopted and the duration of mating was 6, 9, 24 or 36 hours. For 6 and 9 hour mating studies, parental cell densities of 10^5, 10^7 and 10^9 cells/ml were used in a 1:1 donor:recipient ratio. Negative controls were performed and donors and recipients were plated separately on the selection plates. Transconjugant selection plates used were MM + Rif + naphthalene. The donors were enumerated on MM + naphthalene and the recipients on LB Rif plates. The presumptive transconjugants were verified by standard methods as outlined in Section 2.11.1. Stability of the acquired NAH7 plasmid was assessed by repeated transfers of isolated colonies in LB broth and subsequent plasmid isolation.

A 6 hour and a 9 hour filter mating duration was used with an initial parental cell density of 10^6 and 10^9 cfu/ml. Transconjugants were detected at a frequency of $1.5 \pm 0.9 \times 10^{-8}$ transconjugants/recipient after 9 hours of mating, and ~5 presumptive transconjugants were detected on undiluted selection plate after 6 hours of mating only when 10^9 parental cells were used for conjugation. At 30°C, after 24 hours of mating with 10^9 parental cells, transconjugants were observed only on undiluted selection plate showing that conjugation takes place at a very low frequency. With initial parental densities of 10^6 cfu/ml, transconjugants were detected on an undiluted selection plate (ca. 30) only after 24 hours of mating. Similar results were observed with initial parental densities of 10^5 and 10^7 cfu/ml. Low initial parental densities resulted in a low conjugation frequency and transconjugants were detected only after 24 h (Figure 3.9).

The results obtained from this experiment raise the question of whether the number of transconjugants detected after 24 h or 36 h is an inflated value resulting not just from the horizontal transfer of the NAH7 plasmid but also from the vertical transfer of the plasmid during growth of transconjugants on the mating filter. Transfer frequencies are estimated as a ratio of transconjugant cells to donor or recipient cells. Increased transconjugant numbers detected over time might be due to replication of the transconjugants. Hill and Top. (1998) in their review stated that the measured 'transfer frequency' does not reflect the frequency of actual conjugative transfer and use of the term 'transfer frequency' is incorrect. The transconjugant to donor or recipient 'ratio' can still be calculated, but this should be defined as a 'ratio'and be distinct from the true estimate of transfer frequency.

However, the current study proved that the NAH7 plasmid could transfer to another strain by mating on an agar surface. Initial parental cell densities had a direct effect on the number of transconjugants recovered: Lower parental cell densities presumably were not sufficient to achieve cell to cell contact. The intriguing aspect is that these matings were performed on filters to which donors and recipients were directly applied. In spite of the physical closeness of donors and recipients on a small area, transconjugant numbers are low. This might be due to the lower number of potential

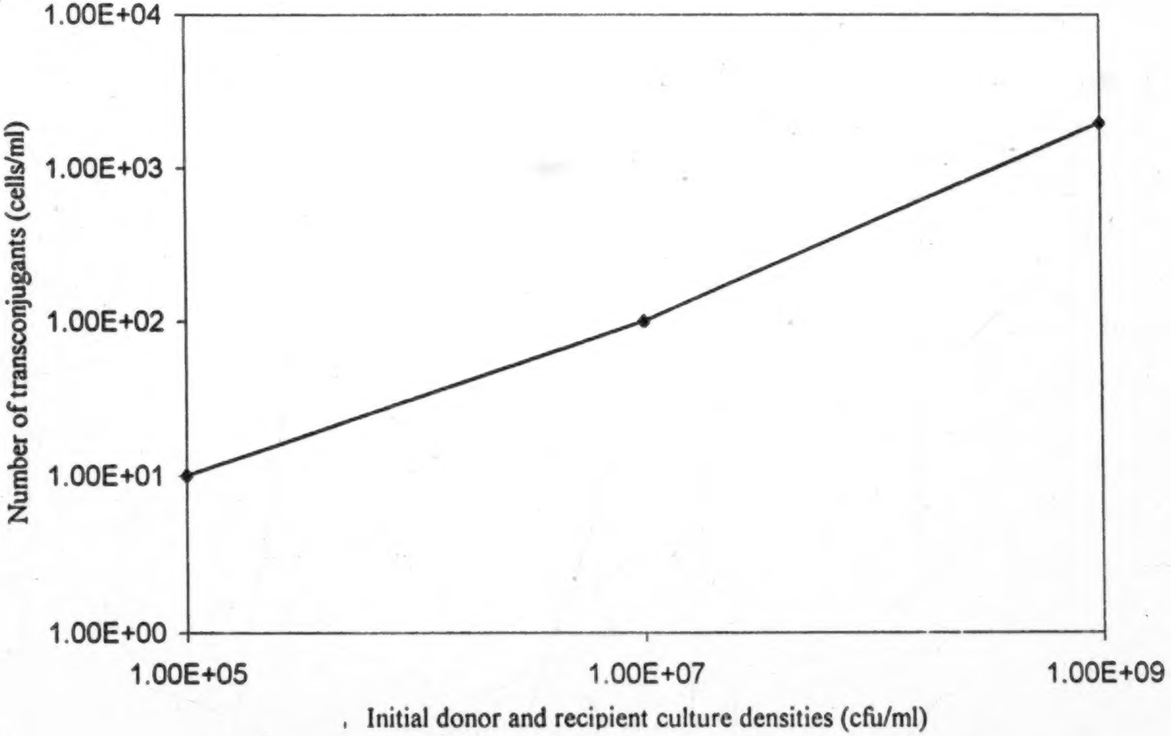

Figure 3.9. Effect of donor and recipient cell densities on NAH7 plasmid transfer by filter mating for 24 h at 26°C.
Values are the means of triplicates.

recipients that can acquire the plasmid. However, the conjugation frequency (i.e. transconjugants/recipient or donor, calculated based on initial parental cell density) was greater when lower cell densities of ~10^5 parental cells/ml were used although the absolute number of tranconjugants was lower. Higher parental densities might mean more donors and recipients could participate in mating pair formation as there would be more recipients and donors i.e. there are potentially more mating pairs. Another factor that needs to be accounted for is the growth of donors and recipients. After 24 hours of mating, donor and recipient cell densities were close to 10^8-10^9 parental cfu/ml, independent of the initial parental cell density used. If conjugation frequency is calculated based on final parental cell densities, then the conjugation frequency is lower when at low initial parental densities. So, one has to be careful when expressing conjugation frequency. Certainly, minimum cell densities are required for mating and 10^5 parental cells might represent the lower limits for conjugal transfer on filters. The transconjugants observed at lower parental densities might arise only after the donor and recipient cell densities increase. This might account for the lack of detectable transconjugants after 6 hours of mating seen at low initial parental cell densities.

3.6. Discrimination between filter mating and mating on selection plates

Mating between the donor and the recipient might have occurred on the selection plate itself i.e. after removal of the mating mixture from the conjugation filter, if the parental cells were not adequately separated during selective plating. The objective of this experiment was to determine if the presumptive transconjugants resulted from true filter mating or were an artefact of subsequent mating on selection plates. This procedure was based on observations by Smit and van Elsas (1990) who reported that temperature influenced conjugation frequencies, since. incubation of mating plates at 4°C reduced the number of transconjugants compared with incubation at 20°C and 27°C. They suggested that the lower metabolic activity of bacteria at colder temperatures might explain the reduced frequency of bacterial mating. They used 4°C preincubation of inoculated selection plates and inclusion of nalidixic acid for donor counterselection, as nalidixic acid interferes with DNA gyrase activity and therefore plasmid transfer, to establish that presumptive transconjugants were not due to mating on the transconjugant selection plate.

P. putida PpG7 (NAH7) was used as the plasmid donor and *P. putida* KT2440Rif^r or *P. putida* KT2440Nal^r as the recipient. Donors and recipients were grown overnight in LB broth without selective pressure and used at ~10^9 cfu/ml, estimated by viable plate count. Standard filter matings were performed on LB plates incubated at room temperature for 18 hours. Parallel sets of selection plates (MM + Rif + Naphthalene) were used: one set was prechilled to 4°C while the other was kept at room temperature (22°C). The 4°C preincubated plates, after inoculation, were maintained for another 3 hours at 4° C before incubation at room temperature. Controls were performed by plating donors and recipients separately on selection plates to check for spontaneous mutations to rifampicin resistance. As a separate test of mating on selection plates, donor and recipient cells were mixed and immediately plated on the transconjugant selection plate.

Few transconjugants were detected when donors and recipients were mixed at the time of plating (2-3 detected/plate). The number of transconjugants seen on selection plates were the same (no significant difference) with and without 4° C pretreatment of the plates (Table 3.2). The data obtained in this experiment showed that illegitimate mating did not occur on the selection plates but occurred on the conjugation filter.

Smit and van Elsas (1990) studied the influence of different antibiotics on donor counterselection and gene transfer. The antibiotics rifampicin, streptomycin, tetracycline, and kanamycin apparently did not inhibit bacteria to the extent at which conjugation of RP4, an antibiotic resistance plasmid, was completely impaired. The expression of the *tra* genes in the donor cells necessary for RP4 transfer is probably constitutive, and therefore conjugation might not be inhibited by the antibiotics since *tra*-gene product levels in donor cells might be sufficient to promote matings. Alternatively, the action of Rif and Sm may be delayed owing to relatively slow penetration into the cells, giving the cells time to conjugate. Rifampicin used for donor counterselection would have inhibited mating on selection plates by lowering the level of *tra* gene products involved in plasmid transfer. Another reason for reduced mating on selection plates could be that the donor and recipient cell density would be low, thereby reducing the possibility of cell to cell contact. When a nalidixic resistant recipient was used, mating on selection plates was not found to occur. Nalidixic acid interferes with DNA replication by inhibiting DNA gyrase activity (Lloyd et al., 1980; Ingraham et al., 1983). Smit and van Elsas (1990) observed a similar effect i.e. no mating on selection plates, when they used a Nal^r recipient.

Conjugal transfer of an antibiotic resistance plasmid between *Burkholderia cepacia* strains resulted in false positive transconjugants on the surface of selective agar plates instead of in microcosms (Walter et al., 1991). Care should be taken when interpreting results from any conjugal transfer experiment, as such matings on selective agar could lead to erroneous and inflated values for the number of transconjugants in

Table 3.2. Number of transconjugants arising from standard filter mating procedure when plated onto prechilled (4°C) selective agar plates or ambient temperature (22°C) agar. Parallel control incubations of parental strain mixtures are also shown. Values are the means of duplicate plates for each of two independent experiments.

	Standard filter mating		**Control incubation of parental strain mixture**	
	Prechilled (4°C)	Ambient (22°C)	Prechilled (4°C)	Ambient (22°C)
Experiment 1	6.5×10^3	9.8×10^3	6×10^0	2×10^0
Experiment 2	4.5×10^3	9.1×10^3	2×10^0	2×10^0

other plasmid transfer systems. The current study helped prove that conjugal transfer in the mating mixture alone resulted in the formation of transconjugants and it was not due to mating on selection plates.

3.7. Kinetics of conjugal transfer of TOL and NAH7 plasmids

The frequency of conjugal plasmid transfer varies significantly and is dependent on a number of factors, e.g., the type of donor and recipient organisms, the plasmid itself and the environmental conditions (Stotzky et al., 1989). The rate of transfer is often orders of magnitude higher on solid surfaces than in liquid suspensions, in particular for plasmids encoding rigid pili (Bradley et al., 1980; 1982). Conjugal transfer events in large populations is taken as the benchmark to estimate bacterial plasmid transfer. This experiment dealt with the estimation of conjugal transfer frequencies of TOL and NAH7 plasmids at various time points on solid agar medium. The transconjugants detected after a certain time period, eg. 12 h or 24 h would include not only transconjugants which were formed due to a primary mating event between the donor and recipient (horizontal transfer) but also from secondary mating events between a transconjugant and another recipient and possibly the progeny from vertical transfer of the plasmid. A time-resolved estimation of conjugal transfer might enable us to determine the dynamics involved in conjugal transfer. There has not been any study on determining the conjugal transfer kinetics of the NAH7 plasmid. The experiments were performed as described in Section 2.6.

3.7.1. Conjugal transfer kinetics of NAH7 plasmid

The first observed transconjugant was detected only after 400 min (approx. 7 h) of mating. Prior to this time, transconjugants might be present, but below the detection limit (<10 transconjugants). Transconjugant numbers increased to 10^3 after 800 min (approx. 14 h) of mating and stabilized until 2000 min (approx. 35 h) of mating. After that, transconjugants increased to a cell density of ~10^5 cfu/mating spot at the end of 2800 min of mating (Figure 3.10).

The initial increase in transconjugants (until 800 min of mating) could be attributed to primary horizontal transfer. However, after 2000 min, any increase in transconjugants might be due additional rounds of primary and secondary mating events. Since there was no growth of recipients during the time period, vertical transfer as a major factor for transfer of the plasmid can be discounted. After initial cell to cell contact between donor and recipient cells and plasmid transfer, the transconjugants might themselves act as donors and bring about subsequent transfer of the plasmid to the recipients. Mating therefore occurred in two waves. Recipient cell density remained constant throughout the duration of the experiment but donor cell density appeared to decrease from 10^9 to 10^7 cfu/mating after 400 min of mating (Figure 3.10). This decrease in donor cell numbers nearly corresponded with an increase in transconjugant numbers. This could be due to non-viability or death of the donor cells or due to aggregation of donor cells to make the viable number appear to decrease. This study showed that vertical transfer might not be accounting for the initial increase in transconjugants but horizontal transfer might be occurring in waves resulting in higher transconjugant numbers.

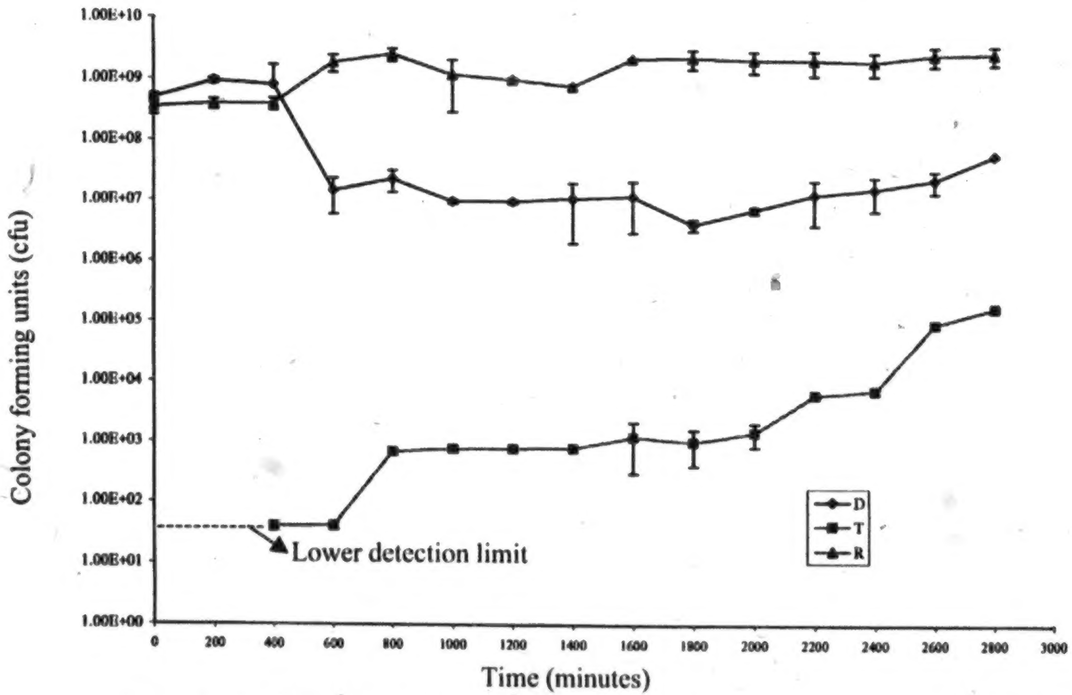

Figure 3.10. Conjugal transfer kinetics of NAH7 during mating of *P. putida* PpG7 (NAH7) to *P. putida* KT2440Rif^r at 28°C on LB agar

Each point represents the mean of triplicates. Error bars where visible indicate ± one standard deviation. D- donors; T- transconjugants; R-recipients

3.7.2. Conjugal transfer kinetics of TOL plasmid

Transconjugants could be detected after 400 min (approx. 7 h) of mating and increased until 1000 min of mating (~10^4 transconjugants) as seen in Figure 3.11. After 1600 minutes of mating i.e. between 26 and 36 h (1600 min - 2800 min), the transconjugant density increased 10 fold and this was maintained for the duration of the experiment (Figure 3.11). Donor and recipient cell numbers remained constant at ~10^9 cfu/mating after 5 h. The initial increase in transconjugants until approx. 16 h (1000 min) might be due to horizontal transfer. The transconjugant cell density stabilizes after that for a short period and the subsequent increase is likely due to a combination of primary and secondary mating events.

TOL plasmid transfer is strongly dependent on the physiological state of the bacteria involved in mating (Smets et al., 1993). An experiment similar to that described here was carried out with *gfp*-tagged TOL plasmid by Christensen et al. (1996) who suggested that TOL plasmid transfer in bacteria on agar surfaces occurs mainly during a short period of time after initial cell to cell contact of donors and recipients. When donor cells (*P. putida* R1/TOL*gfp*mut3b) and isogenic recipient cells (*P. putida* R1) were mixed in equal numbers on an agar surface, approximately 20% of the recipients hosted the plasmid after 24 h (Christensen et al. 1998). The current study differed from the work of Christensen et al. (1996) in the preparation of donor and recipient cells and the medium used. In this study with TOL and NAH7 plasmids, donor and recipient cells were not centrifuged prior to mating to avoid mechanical shearing of pili and so conjugal transfer would not have been adversely affected.

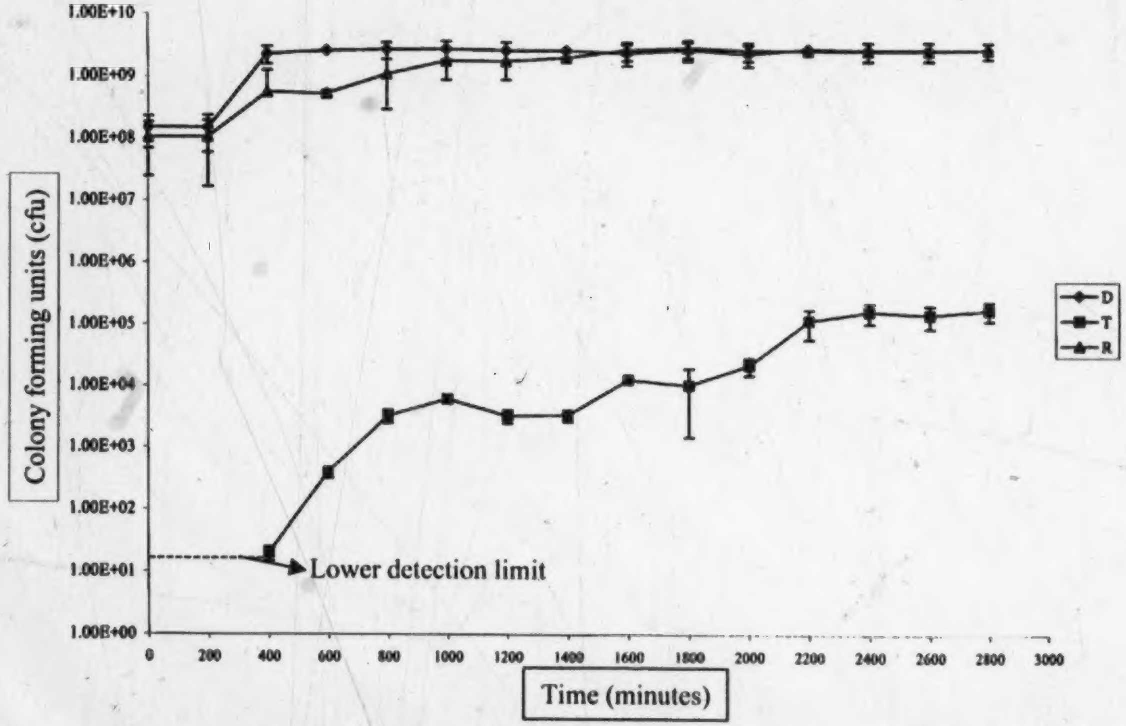

Figure 3.11. Conjugal transfer kinetics of TOL during mating of *P. putida* ATCC33015 (TOL) to *P. putida* KT2440Rif^r at 28°C on LB agar.

Each point represents the mean of triplicates. Error bars where visible indicate ± one standard deviation. D-donors; T-transconjugants; R-recipients.

This experiment demonstrated that transfer frequencies of TOL and NAH7 estimated after 800-1000 min (14-16 h of mating) might not give a true indication of primary horizontal transfer only. Since donor and recipient cell densities remained constant throughout the duration of the experiment, it can be assumed that only horizontal transfer is the major mechanism of plasmid transfer. The above results might be valid only with agar surface mating, where there is intimate cell to cell contact and high donor and recipient cell densities. This experiment also shows the necessity of distinguishing between the total number of transconjugants observed after a longer time period (as it would include the sum of primary and secondary horizontal transfers and possibly vertical transfer) and transconjugants observed after a shorter mating period (where the possibility of inflated transconjugant numbers is limited). Sampling time after inoculation will affect transconjugant numbers and any subsequent interpretation of these counts. Hill and Top (1998) stated that increases in transconjugant numbers with time are likely to result from replication of the initial transconjugants, particularly in instances where selective pressure actively promotes growth of these transconjugants.

Transconjugant numbers compared well to the recipient numbers when grown alone (data not shown) but it should be noted that when grown alone, competitive effects i.e. between donors, recipients and transconjugants are not in play.

3.8. Effect of Donor:Recipient ratios on conjugal transfer frequencies

The objective of these experiments was to determine if conjugal transfer frequencies of TOL and NAH7 plasmids are affected by various ratios of donor to recipient cells. If recipients are present at a higher proportion than the donors, conjugal transfer frequency might be enhanced because there should be sufficient recipient cells for each and every donor to establish a mating pair and transfer the plasmid.

NAH7 and TOL plasmids were used for the study. Standard agar plate matings were carried out (see Section 2.4.2). Donor and recipient cells, grown overnight in LB, were used for the mating experiments. Ratios of 1:5 and 1:10 (donor:recipient cells; D:R) were compared with a 1:1 ratio. Plates were incubated at 28°C for 24 hours and transconjugants were selected on appropriate selection medium. Plasmids were isolated from randomly selected presumptive transconjugants to verify plasmid acquisition.

A six fold higher conjugal transfer frequency [(1.2 ± 0.8) x 10^{-5} transconjugants/recipient] was observed both with a 1:5 and a 1:10 D:R ratio when compared to a 1:1 ratio [(2.0 ± 0.6) x 10^{-6}]. The higher transfer frequencies observed might have resulted from the transconjugants acting as donors in combination with the original plasmid donors. The observed frequency of transconjugation might include not just the transconjugants arising from the initial transfer of the plasmid into the recipient, but also from subsequent transfer from transconjugant to a new recipient, and possibly vertical transfer. Christensen et al. (1998) stated that vertical TOL plasmid transfer was dependent on the existing pool of recipient *P. putida* R1 cells; the size of this pool significantly influenced transconjugant establishment and the introduction of new genetic traits by plasmid transfer.

Different donor and recipient cell densities also influence conjugal transfer. Christensen et al. (1998) followed transfer of a TOL plasmid derivative from a donor provided at concentrations of 10^6, 10^7 and 10^8 cfu/ml, and found that higher donor cell densities yielded higher initial conjugal transfer. Thereafter, the nutritional advantage of the resulting transconjugant cell allowed the transconjugants and their progeny to grow quickly and produce microcolonies. They also observed that, although transconjugant and recipient cells seemed to be tightly associated in the microcolonies, horizontal transfer of the TOL plasmid through the entire recipient colony was never observed. Generally, over a longer period i.e. 15 to 20 days, transconjugant numbers stabilized which could be due to a combination of factors including vertical transfer, transconjugant die-off, competition and reduction in nutrient levels.

High population densities are required for successful genetic exchange. At low population densities the probability of successful genetic exchange is reduced and the process generally is not significant for natural bacterial population densities, as greater than 10^5 cfu/ml are generally required to observe such genetic exchange by conjugation (Atlas and Bartha, 1981). Higher donor:recipient densities and ratios would in fact be contributing both to horizontal and vertical transfer, as successful establishment and adequate cell densities of donor and recipients are required for successful plasmid transfer.

3.9. Microcosm mating

3.9.1. Transfer of NAH7 in sterile sand microcosms with and without selective pressure

The aim of this section was to demonstrate transfer under simulated environmental conditions in three dimensions as opposed to two-dimensional agar surface conditions described earlier. Microcosms serve as valuable tools in the study of horizontal gene transfer. Hence the conjugation frequency of the NAH7 plasmid in a sterile sand microcosm was estimated using *P. putida* PpG7 (NAH7) as the donor and *P. putida* KT2440Rif^r as the recipient. The results are discussed in Section 3.9.1.2 and 3.9.1.3.

3.9.1.1. Validation of sampling technique

The sampling technique adopted for beaker microcosms was verified for its appropriateness and representative nature. In the subsampling method used here, 3.1 g samples of sand were taken aseptically from different parts of the microcosm. It remained to be demonstrated however, whether this subsample adequately represented plasmid transfer events taking place in the whole microcosm or whether there were heterogeneous pockets in the microcosm where the transfer frequency varied. This concern was addressed by sacrificing entire microcosms (in dilution bottles) and comparing the results with subsamples taken from parallel beaker microcosms.

A 1:1 donor to recipient ratio of *P. putida* PpG7 (NAH7) and *P. putida* KT2440Rif^r gave a conjugal transfer frequency of $>10^{-7}$ transconjugants/recipient regardless of the sampling technique. This experiment showed that the standard subsampling technique is accurate and representative of the entire microcosm.

3.9.1.2. Microcosm matings without nutritional selection

Matings were carried out in sand microcosms at 26°C and 30°C by mixing parental strains and LB broth and adjusting the mixture to 23% (wet weight) as described in Section 2.8.1. After 24 hours of incubation, samples were taken aseptically from different areas of the microcosms, diluted, and plated appropriately on medium selective for transconjugants. Samples taken from mating microcosms contain not only transconjugants, but also donors and recipients, and there is the possibility that conjugation would occur on the selective media (Walter et al., 1991). To test this possibility, samples from parallel microcosms inoculated with either donors or recipients were mixed and immediately plated on transconjugant selection plates.

Since LB was used as a nutrient source in the microcosm, this likely supported the growth of the organisms during incubation. Transconjugants were not detected in microcosms incubated at 30°C (detection limit is ca. 10 transconjugants). Although donor and recipient cell densities were high (Table 3.3), only 20 presumptive transconjugants were detected on undiluted selection plates when microcosms were incubated at 26 °C (room temperature). All the presumptive transconjugants were replica plated on MM + Rif + naphthalene. Only a few colonies (ca. 11) grew and these colonies harbored the NAH7 plasmid. These colonies might be spontaneous Rif^r mutants of the donor or *P. putida* KT2440Rif^r might have grown on the original selection plate due to carryover of LB from the microcosm. Similar growth was also observed on donor and recipient control plates. The frequency of transfer of the NAH7 plasmid might be too low to detect in the absence of selective pressure in the microcosm.

Top et al. (1990) showed that the mobilization of the IncP plasmid pDN705 from *Escherichia coli* to *Alcaligenes eutrophus* was detected in a sterile sandy soil at 20°C only when nutrients were added (5×10^{-7} transconjugants/recipient after 4 days mating). In a richer sterile sandy loam, transfer occurred at similar frequencies in both nutrient-amended and unamended soil (1×10^{-5} transconjugants/recipient after 1 day mating) but

Table 3.3. Donors, recipients and transconjugants detected in microcosms after 24 h incubation at room temperature (ca. 26°C)

Strains enumerated	Cell densities (cfu/g wet weight sand)[a]
P. putida PpG7 (NAH7); donor control[b]	$2.8 \pm 0.9 \times 10^8$
P. putida KT2440Rif^r; recipient control[b]	$9.4 \pm 1.0 \times 10^7$
Combined parental strains on non-selective medium	$2.1 \pm 0.6 \times 10^8$
P. putida KT2440Rif^r (NAH7); presumptive transconjugants	$2.0 \pm 0.6 \times 10^1$

[a] Mean of triplicate values ± standard deviation

[b] Donor and recipient cells grown independently in microcosms enumerated on selective medium

no transfer was observed in non-sterile soil unless it was nutrient amended. A nutrient-rich habitat generally results in growth of organisms, thus facilitating cell to cell contact.

The ability of bacteria to sense and swim toward aromatic hydrocarbons is an important prelude to their degradation. Grimm and Harwood (1999) showed that the presence of the *nahY* gene in NAH7 facilitated chemotaxis. This would imply that, in the current study, in the absence of selective pressure, donors might not migrate and aggregate at particular areas, thereby reducing the chances of donor-recipient contact, even though the recipients lack the chemotactic response.

Effective migrations of bacteria through soil micropores would occur only at moisture contents equal to field capacity. There might not be rich pockets of donors and recipients facilitating gene transfer. The sand microcosm, in spite of bearing a close resemblance to plate mating (in terms of parental cell numbers) did not support high conjugal transfer. This might be because of spatial dispersal of the donors and recipients. Tamanai-Shacoori et al. (1995) showed that the transfer frequencies of antibiotic resistant plasmids in *E. coli* in raw and untreated waste water, though a different substrate, were several orders of magnitude below that observed in LB medium but they explained the decrease in transfer frequencies as being due to stress caused by abiotic environmental factors. Actually a combination of biotic and abiotic factors could be operating in such a complex ecosystem as wastewater.

An important factor which might affect plasmid transfer and plasmid maintenance in a natural environment is the presence of selective pressure. Natural microbial communities can respond to environmental stress by enrichment of those microorganisms in the community that tolerate stress conditions or that more rapidly degrade pollutants (Barkay and Pritchard, 1988).

This experiment was carried out in parallel on agar plates with and without nutritional selection but there was no significant difference in conjugation frequency. This could be due to the presence of donors and recipients on the small filter area instead of being distributed in a larger microcosm and also bound to the sand. In microcosms, this would lead to lower conjugal transfer. Another possibility could be the poor recovery of true transconjugants from sand after mating has occurred. The vortexing procedure might not have dislodged all transconjugants adhering to the sand particles.

Plasmid transfer should ideally occur at high frequencies in nutrient rich habitats owing to high donor and recipient cell densities but that does not seem to be the case in our experiment. The current study showed that transfer of the NAH7 plasmid remained undetected in the absence of selection pressure in sterile sand microcosm.

3.9.1.3. Microcosm mating with nutritional selection

Organic carbon sources have been shown to enhance the frequency of plasmid transfer. The objective of this experiment was to determine whether NAH7 plasmid transfer in a sterile sand microcosm could be detected when nutritional selective pressure was applied through the addition of naphthalene. *P. putida* PpG7 (NAH7) and *P. putida* KT2440Rif^r were used as donor and recipient in standard sand microcosms.

Transconjugants at a density of 4 ± 0.9 x 10^2 transconjugants/g of sand wet weight were isolated from microcosms when naphthalene was supplied as crystals. This corresponded to a conjugation frequency of 10^{-7} transconjugants/recipient. Tiny colonies causing yellowing of the agar were seen on undiluted selection plates when microcosms were provided with naphthalene dissolved in diethyl ether. No growth was observed on donor or recipient control plates. After 8 hours of mating, in naphthalene-amended microcosms incubated at room temperature, only ca. 18 presumptive transconjugants were detected on the undiluted selection plate. When microcosms with naphthalene supplied in diethyl ether were incubated at 30°C for a mating period of 24 hours, ca. 20 presumptive transconjugants were recovered. Plasmids were isolated from presumptive transconjugants (Figure 3.12). The insertions in NAH7 and the stability of these plasmids are discussed in Section 3.4. Restriction digestion profiles of these plasmids resembled those of the transconjugants obtained by filter mating (see Section 3.4, Figure 3.5) and appear to represent NAH7 with duplications.

There are several possible reasons for the low number of transconjugants isolated from microcosms supplied with naphthalene in diethyl ether. The residual diethyl ether itself might have been toxic to the microorganisms. Naphthalene supplied in diethyl ether (7.5 mmoles/ml) might have been too toxic to the organisms (parental cell densities of 10^6 cfu/g of sand were recovered from these microcosms compared with initial inoculum densities of 10^9 cfu/g sand). This parental cell density was probably too low to bring about cell to cell contact of donors and recipients in the microcosm. When naphthalene was provided as crystals, there might have been areas of high and low naphthalene concentrations. Fewer plasmid transfers might have taken place in areas with lower donor and recipient cell densities which might be due to naphthalene toxicity. Conjugation would have occurred in regions where the naphthalene concentration was not too high as these areas might have supported a higher population to bring about cell

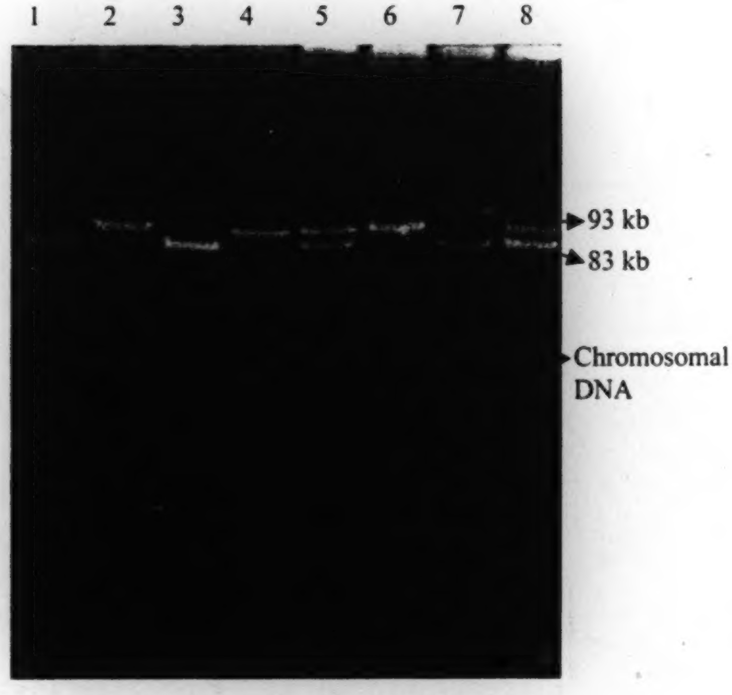
1
2
3
4
5
6
7
8
93 kb
83 kb
Chromosomal DNA

Figure 3.12. Agarose gel containing NAH7 plasmid and its derivatives isolated from transconjugants after sand microcosm mating.
Lane:1, NAH7 (83 kb); lanes 2-8, Transconjugants (pKTN series)

to cell contact and thereby transfer of the NAH7 plasmid. The concept for a maximum or upper threshold concentration for microbial degradation of hydrocarbons might apply also to soil ecosystems (Leahy and Colwell, 1990). Therefore, in the current study a toxicity limit might be in operation and this could influence conjugal transfer indirectly by inhibiting parental and transconjugant growth.

Similar studies of TOL plasmid transfer indicated a positive correlation between plasmid transfer in sand microcosms and the presence of *m*-toluate as the selective pressure. Without selection pressure, conjugal transfer frequency of $1.5 \pm 0.6 \times 10^{-8}$ transconjugants/recipient was observed whereas with selection pressure, higher conjugation frequency was noted ($2.0 \pm 0.4 \times 10^{-7}$ transconjugants/recipient).

Hill and Top (1998) in their review stated that nutritional selection in a soil habitat can strongly affect the extent of gene spread and hence needs to be considered as an influential factor. Exposure of natural microbial populations to crude oil or other hydrocarbons might impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community (Leahy and Colwell, 1990). This increased plasmid frequency could result in more plasmid transfer events to different suitable recipients. Neilson et al. (1994) found that transfer of pJP4 from *A. eutrophus* JMP134 to *Variovorax paradoxus* in nonsterile soil amended with 100 ppm 2,4-D occurred at 10^{-6} per parent strain after 48 hours compared with unamended soil.

On a conflicting note, Ramos-Gonzalez et al. (1991), based on their transfer studies with the catabolic plasmid pWW0-EB62 in *Pseudomonas* in sterile soil, reported that the addition of *p*-ethylbenzoate as selective agent caused a delay in the appearance of transconjugants, probably caused by die-off of the recipient strain. Cell densities in contaminated sites might be insufficient to bring about plasmid transfer by a passive cell to cell contact process requiring high cell densities whereas selective pressure might provide the impetus for the transfer events. The results obtained in this study also showed that selective pressure could affect transfer of the NAH7 plasmid.

Duncan et al. (1995), in their study on NAH7 plasmid transfer, reported that the greatest development of a transconjugant population occurred in microcosms that did not contain naphthalene, and hence the enrichment was not directly due to a selective advantage. However their experiment was carried out in microcosms which contained organic carbon in addition to naphthalene and the pH of the medium was only 4.6. Besides, the naphthalene crystals that were still visible at the end of the experimental period might have led to toxicity problems and interfered with accurately interpreting the effect of selection pressure on NAH7 plasmid transfer. In our experiment, the microcosm pH was close to 7 and naphthalene crystals might not have posed a toxicity problem because only ~1 g of naphthalene crystals were used when the crystals were added directly to the microcosm.

The original function of degradative plasmids might have been to detoxify hydrocarbons, not to use them as a source of energy (Farrell and Chakrabarty, 1979). In the absence of an alternative source of energy, the organisms might have evolved to use the hydrocarbon. The underlying hypothesis of evolution, i.e. that selection pressure drives the evolutionary process, is also favorably addressed in the current study. In spite of the toxic effect exerted on the recipient (as demonstrated by low recipient recovery), which cannot utilize naphthalene, conjugal transfer frequency can be detected because the recipient may utilize the metabolites produced by the degraders (the donor and the transconjugants). In nature, such selective pressures might influence a number of microorganisms, thereby necessitating their acquisition of the degradative plasmid. This might explain the diversity of microorganisms harboring similar degradative plasmids. This also raises the hope that introduction of a donor with a catabolic plasmid would be a viable strategy for bioremediation, as selective pressure would drive the process of plasmid transfer. On an alternative note, even if transconjugant densities alone do not increase to effectively stimulate bioremediation, they could serve as intermediaries in the process of evolution of organisms with greater degradative potentials.

De Rore et al. (1994) showed that conjugal transfer of pDN705, encoding resistance against cadmium, zinc and cobalt, occurred between *E. coli* and *Alcaligenes eutrophus* in heavy metal polluted soil. As filter matings showed no effect or even a negative effect of Zn^{2+} on plasmid transfer, the increase in the number of transconjugants in polluted soils was probably due to growth rather than stimulated transfer. Wickham and Atlas (1988) found that changes in catabolic plasmid frequency in bacterial populations were dependent on the amount and the type of chemical stress. In contrast, Lawlor et al. (1999) reported that the number of putative donor and recipient colonies observed on selective agar was not affected by the concentration of metals in the soil, nor were the number of transconjugants. However, there were differences in transfer frequencies of mercury and copper resistance from the different soil samples.

An interesting point here is that, in spite of donor and recipient densities being similar in microcosms with and without selection pressure, higher numbers of transconjugants were observed only with selective pressure; it might not be just cell to cell contact that is essential for plasmid transfer. Herein lies the evolutionary significance of gene transfer to counter the adverse effects that might arise in the environment. The process of horizontal plasmid transfer is fuelled by a number of physical, chemical and biological factors in the environment. NAH7 might even confer additional cryptic advantages to its hosts in soil environments and the plasmid might, in fact, transfer to more hosts so that it has a greater chance to persist even without selection pressure. Alhough the typical sand microcosm model used here does not exist in nature, this experiment showed the necessity of selective pressure to accelerate the process of horizontal transfer of this catabolic plasmid.

Walter et al. (1991) reported that conjugal transfer of an antibiotic resistance plasmid in soil might have continued past day 2, but because the donor population also decreased over time, the conjugal transfer rate might have fallen below the death rate of the transconjugant population. In such a case, one must consider that a highly fit transconjugant could then perpetuate by vertical transfer. The sand used in the microcosms offer a very porous environment but in the environment, factors such as soil pore sizes, microbial cells sizes and attachment of cells to the soil might result in reduced cell transport and thereby reduce cell to cell contact. A number of other interactions also occur in natural ecosystems and the introduced donor might displace potential but not well-adapted recipients from the ecosystem and thereby affect other essential soil and nutrient cycles that operate in the environment. In the current study, in spite of selective pressure, horizontal transfer did not lead to high transconjugant numbers but after a longer period vertical transfer might lead to successful establishment of the transconjugants. Stotzky and Babich (1986) reported that the ability of microbes to maintain high numbers and to migrate through soil depends on the characteristics of each species and is an important factor in the process of genetic transfer. Genetic transfer will be limited to the area of dispersal of donor and recipient strains and has been shown to be primarily influenced by the amount of water present which aids mobility and survival of cells (Lafuente et al., 1996). Therefore, the experiment described here should also be carried out under a series of different moisture regimes.

Although sterile sand microcosms are less than ideal models of a soil environment, studies using sterile microcosms provide a starting point for analyzing horizontal transfer of these large plasmids and this experiment showed the positive effect of selective pressure on the transfer of large catabolic plasmids.

3.9.2. Effect of moisture content

Different moisture contents were imposed on sand microcosms to test the effect of moisture on conjugal transfer of NAH7 plasmid from *P. putida* PpG7 (NAH7) to *P. putida* KT2440Rif^r. Moisture contents ranging from 20 to 23% (on wet weight basis) yielded the most transconjugants ($1.5 \pm 0.5 \times 10^3$ transconjugants/g sand) in our study. Low (10%) and high (30%) moisture content seriously affected conjugal transfer of the NAH7 plasmid ($8.0 \pm 0.3 \times 10^1$ transconjugants/g sand at 10% moisture; $2.0 \pm 0.35 \times 10^2$ transconjugants/g sand at 30% moisture content). Parental cell densities recorded was lower at 10% (100 fold lower compared to 20-23% and 30% moisture content).

Lafuente et al. (1996), in their studies on conjugal transfer of plasmids between *Rhizobium meliloti* strains and from *E. coli* to *R. meliloti,* found that 20% moisture resulted in maximal conjugation frequencies, while soil moisture higher than 20%

decreased the conjugation frequency. The surface area available for mating events in the microcosms depends on the dispersal of the microorganisms in the microcosm which is affected by moisture content. For example, Stotzky and Babich (1986) found that genetic transfer by conjugation depended directly on the possibility of cell to cell contact and that the percentage of water applied over the soil surface was a key factor in both the mobility and survival of the cells.

Low soil moisture content might disrupt the continuity between soil pores and thereby interfere with cell migration. This in turn would reduce cell to cell contact and inhibit conjugal transfer. Low soil moisture content might also impose a moisture stress on the organisms. Though water facilitates movement of the donors and recipients, moisture contents above 23% did not yield higher transconjugant densities in our study on the conjugal transfer of NAH7. Lafuente et al. (1996) hypothesised that the lower transconjugant numbers observed at moisture contents above 20% could be affected by characteristics of the conjugative pilus encoded by plasmid RP4. It is rigid and inflexible, and suitable cell contact to permit plasmid transfer is achieved only if the cells are adsorbed onto a solid surface. They found that, when moisture content is high, appropriate contact between the *E. coli* cells could not be maintained throughout the transfer process. A similar effect might be involved in the reduced conjugation frequency of the NAH7 plasmid observed here in higher moisture regimes. On a contradictory note, Richaume et al. (1989) reported that a moisture content of 8% (a moisture content less than field capacity) produced the maximum transfer frequency when they studied transfer of pBLK1-2 from *E. coli* to *R. fredii*, whereas the highest numbers of surviving donors and recipients were recovered at 20% moisture.

Alexander (1977) indicated that maximum bacterial density is found in soils of fairly low moisture content, and the optimum moisture level for the activities of aerobic bacteria is from 50 to 75% of the moisture holding capacity of the soil. To optimize conjugation, the moisture content should be adequate to support a sufficient bacterial density to bring about cell to cell contact. In soil, though water provides the matrix for cell to cell contact, it might also not be the sole factor involved in enhancing conjugal transfer as this is also dependent on the type of conjugative pilus encoded by the plasmid.

3.9.3. Effect of microcosm disturbance

The objective of this experiment was to demonstrate the influence of the substratum on conjugal transfer. It was expected that the type of microcosm used (i.e. jars or packed columns) and whether the sand was disturbed or compacted would have an effect on conjugation frequencies (Hill and Top, 1998). The potential for gene transfer in the soil environment depends on the survival and transport of organisms in the ecosystem (Trevors et al., 1987). This experiment showed that a slight difference in microcosm design could lead to different observed transfer frequencies.

P. putida strains PpG7 (NAH7), KTN4 (+NAH7 and NAH7) and KTN (NAH7) were used as plasmid donors and KT2440Nal^r used as the recipient in 1:1 D:R ratios. Microcosms were set up either as compacted sand microcosms (undisturbed after inoculation) or as disturbed microcosms (mixed after inoculation); see Section 2.8.5. Transconjugants, donors and recipients were enumerated on appropriate selection plates. The experiment was performed twice in duplicate.

In the disturbed sand microcosm, a conjugation frequency of $2.8 \pm 0.9 \times 10^{-8}$ transconjugants/recipient was observed whereas higher conjugal transfer frequencies were observed in compacted sand microcosms ($3.5 \pm 0.8 \times 10^{-7}$transconjugants/recipient) and donors did not influence conjugal transfer. The compacted sand microcosm might approach the spatial conditions on a plate mating or it might be even better, owing to the selective pressure in the microcosm in contrast to non-selective conditions during plate matings. Disturbing the sand microcosm might decrease the probability of donor and recipient contact, thereby impeding transfer rates. In most studies on bioremediation in microcosms, the success depended on the fact that penetration or mixing of the bacteria with the body of soil occurred to an extent that the organisms encountered much of their substrate (Alexander, 1994). The same reason could also drive the process of gene transfer in microcosms.

In this experiment, donor and recipient cell densities might be low in the microenvironments in the disturbed microcosms (thereby reducing the likelihood of cell to cell contact), whereas in the compacted microcosm, the cells could be retained by the sand matrix. The chance of donor and recipient cells coming into contact could be higher in the compacted microcosm, increasing the conjugation frequency. As well, in compacted microcosms, the pores are not disturbed, thereby permitting cell migration. Smith et al. (1985) suggested that soil structure affected the degree of *E. coli* cell transport because mixed repacked soils retained cells more efficiently (93%) than undisturbed intact soil (21-78% retention).

Since the microcosms in the current study lack an active leaching process, reduced cell to cell contact might be the major factor accounting for lower transconjugant numbers in disturbed microcosms. The repacking of the sand might have enabled compaction of the sand compared to the intact sand. A disturbed microcosm might not be favorable for establishing a stable population of mating donors and recipients. Movement of air and water is also important in importing and distributing nutrients for microbial growth and in the removal of metabolic by-products. Atlas and Bartha (1981) stated that the movement of materials in and out of ecosystems is controlled by factors such as solubility, diffusion, viscosity, specific gravity, porosity and the flow characteristics of the ecosystem. The pores or interstitial spaces determine the diffusion or movement of the microbes along with the solute and if the pores are disturbed, then movement might also be hindered. This experiment was also repeated in dilution bottle microcosms (see Section 2.8.4) so that the entire microcosm could be sacrificed and transconjugants enumerated. Disturbed and compacted dilution bottle microcosms repeated the results observed with the beaker microcosms.

3.9.4. Effect of pH

Soil variables play an important role in influencing plasmid transfer. Soil pH will have an effect on the growth of microorganisms and the solubility of cations which might influence growth of microorganisms. Several studies have found pH to be important in determining *in vitro* transfer frequencies (Krasovsky and Stotzky, 1987; Richaume et al., 1989). The objective of this experiment was to determine the influence of pH as a factor in the horizontal transfer of NAH7 plasmid to a known recipient.

P. putida strains PpG7 (NAH7) and KT2440 Rif^r were used as donor and recipient respectively at a 1:1 ratio. Matings were performed in standard sand microcosms. The interstitial fluid pH was adjusted using mineral medium buffered at pH 5.8, 6.3, 6.6, 7.0, 7.2 and 7.6 using phosphate buffer (see Appendix B).

At pH 5.8, transconjugants were not observed (Figure 3.13). The lower detection limit for transconjugants was <10^2 cfu/g of sand wet weight. Transconjugant numbers increased in the range of pH 6.6 – 7.2. This paralleled the high donor and recipient cell densities recovered at this particular pH range. At lower pH values, recovered donor and recipient cell densities were very low.

Richaume et al. (1989) stated that reduced transfer frequencies from *E. coli* to *Rhizobium fredii* at lower soil pH were probably due to the reduced number of viable donor and recipient cells, which were not detectable in soil below a pH of 5.25. The soil pH might have an effect on plasmid transfer only by reducing donor and recipient cell viabilities such that cell to cell contact is limited. The pH also influences the dissociation of many molecules which indirectly influence microorganisms (Atlas and Bartha, 1981). Krasovsky and Stotzky (1987) confirmed that maximum transfer frequencies of plasmids between *E. coli* occurred at a soil pH of approximately 7.0. Richaume et al. (1989), using an intergeneric model system i.e. *E. coli* and *R. fredii* as donor and recipient respectively, found that the highest plasmid transfer frequency was obtained at a soil pH of 7.25. The reason for higher transfer frequencies at neutral pH could be because bacteria generally favor such pH regimes. Lafuente et al. (1996) in their studies on plasmid transfer with *E. coli* and *R. meliloti*, reported that at pH below 6, the numbers of donor and recipient cells recovered from microcosms were reduced and transconjugant cells were efficiently recovered between pH 7.5 and 8. They also stated that higher pH reduced the viability of the transconjugant cells.

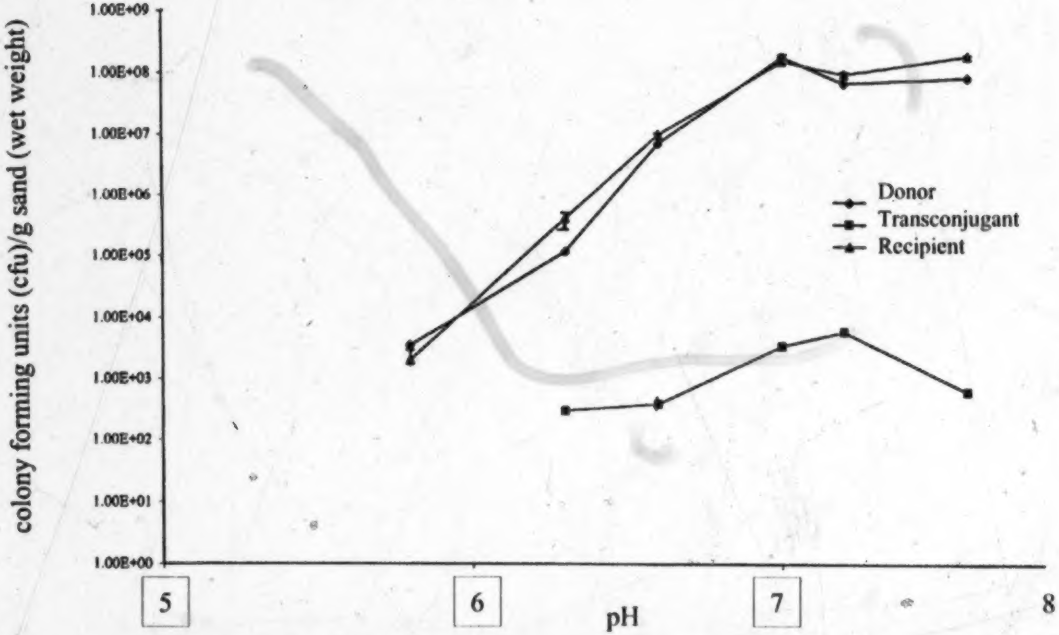

Figure 3.13. Influence of pH on NAH7 plasmid transfer in sand microcosms incubated at 25°C for 24 h. Each point represents the mean of triplicates. Error bars where visible indicate ± one standard deviation

In natural ecosystems, there could be different organisms adapted to different pH conditions and the dynamism of plasmid transfer could be different. There might not be any stable one to one interaction between two organisms that prefer similar pH conditions as shown in this experiment. Hence plasmids might still be transferred to well adapted organisms which can thrive at non-neutral pH. Therefore, the influence of pH on conjugal transfer is mainly organism-dependent. It has not been shown that pH interferes with plasmid transfer machinery in a direct fashion by interfering with the conjugal transfer machinery. In effect, pH might be a major factor influencing plasmid transfer in soil ecosystems compared to aquatic and well-buffered soil ecosystems.

3.9.5. Influence of temperature

Physicochemical and biological characters of the soil affect conjugal transfer. Temperature might play a major role in conjugal transfer by affecting not only the survival of donors and recipients but also by influencing the conjugation process itself. Temperatures higher or lower than the growth optimum modify the physiological state of the bacteria (donor and recipient used) and thereby the plasmid transfer might be affected.

The objective of this experiment was to determine the optimum temperature for conjugal transfer of TOL and NAH7 plasmids in sand microcosms. *P. putida* strains PpG7 (NAH7) and ATCC33015 (TOL) were used as plasmid donors and KT2440Rif^r was used as the recipient. Microcosms (1:1 D:R ratio) for studying NAH7 plasmid transfer were supplemented with naphthalene crystals and incubated at 10, 20, 25 or 30°C for 24 and 48 h. A 1:5 donor to recipient ratio (10^9 cells/ml) and incubation temperatures of 10, 20, 25, 30 or 35°C were used to study TOL plasmid transfer in microcosms supplied with *m*-toluate dissolved in mineral medium. Standard sampling and appropriate selection media were used.

Optimum transfer of NAH7 was observed at 25°C but transconjugants were still detected at 10°C albeit at low frequencies (Figure 3.14). Conjugation frequency after 48 h resembled the trend observed after 24 h of mating but the increase in transconjugants could be due to primary and secondary rounds of horizontal and vertical transfers. In the current study, we found that recovered donor and recipient cell densities were 10 fold lower at 10°C than at 25°C (i.e. recoverable cfu in control microcosms after 24 hours were ~10^7 cfu/g of sand on wet weight basis). The lower frequency of transfer observed at low temperature therefore could be due to a combination of factors including reduced cell to cell contact, modified physiological state of the bacteria or due to an effect on the plasmid transfer machinery. The parental strains are important here since it is their membrane fluidity and cold tolerance/temperature tolerance that will affect both horizontal and vertical transfer.

Temperature also influenced TOL plasmid transfer with the optimum transfer being detected at 30°C (Figure 3.15). Transconjugant frequencies observed after 48 h did not increase significantly from those observed after 24 h, except at 10°C and 35°C, which could be due to substrate limitation or due to competition from the parental strains. A detectable conjugation frequency was observed at 10°C. This could be because the donor to recipient cell density might be sufficient to bring about cell to cell contact for plasmid transfer. Another reason could be because of the 1:5 donor to recipient ratio used for TOL plasmid transfer (compared with a 1:1 ratio for NAH7). The initial transconjugants

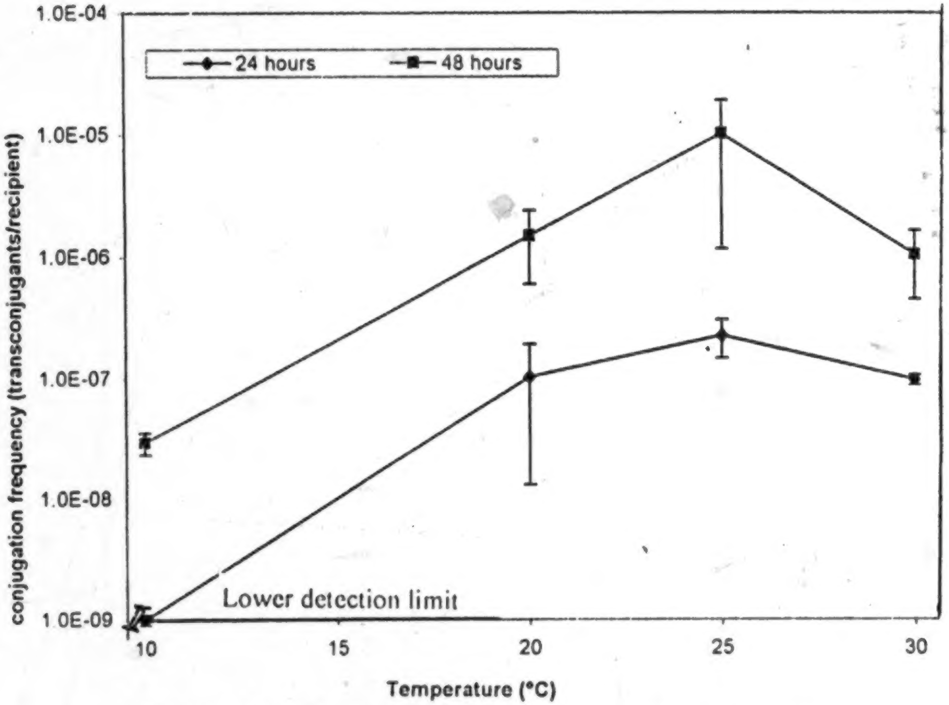

Figure 3.14. Influence of temperature on NAH7 plasmid transfer during 24 or 48 h incubation in sand microcosms.

Each point represents the mean of triplicates. Error bars represent standard deviation.

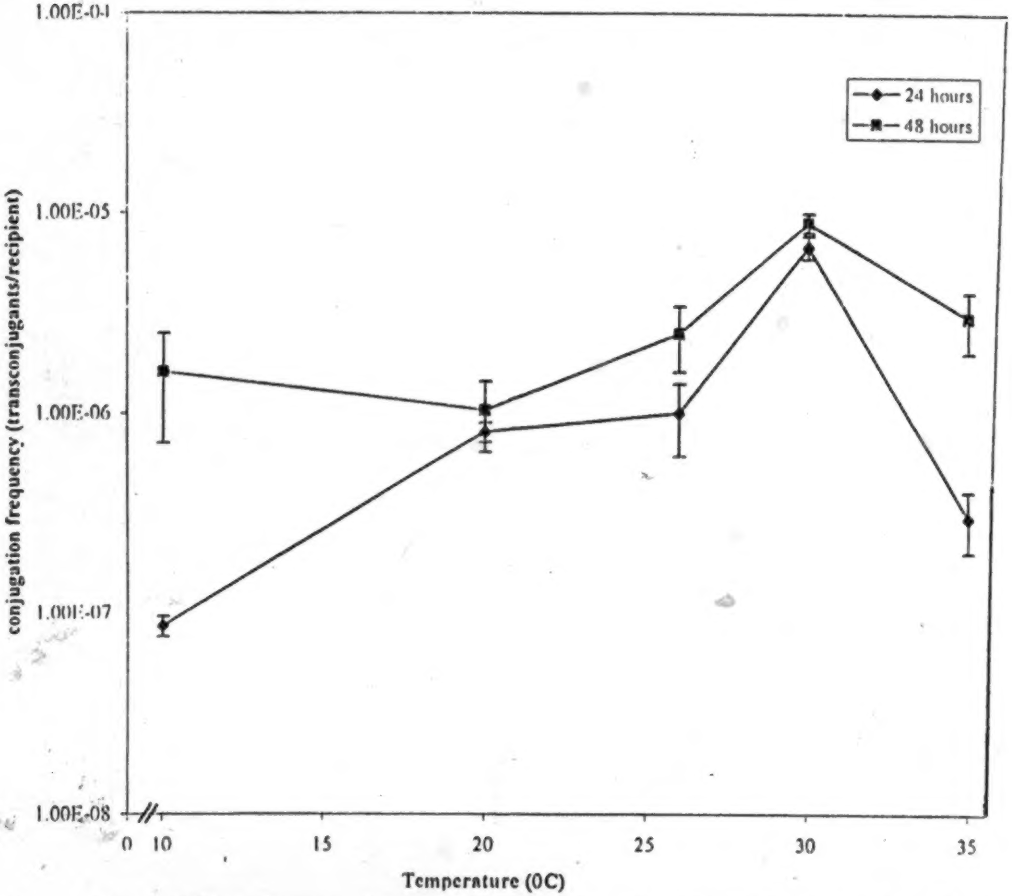

Figure 3.15. Influence of temperature on TOL plasmid transfer during 24 or 48 h incubation in sand microcosms.

Each point represents the mean of triplicates. Error bars represent standard deviation.

formed might be acting as donors, subsequently resulting in a higher conjugation frequency.

Walmsley (1976) showed that mating-pair formation in *E. coli* occurred between 24°C and 45°C with an optimum ca. 41°C. Trevors and Oddie (1986) reported that antibiotic resistance plasmids were transferred between *E. coli* strains in LB-amended sterile soil incubated at 15°C.

The optimum temperature for transfer of NAH7 (25°C) is rarely achieved in the bulk phase of temperate soils and so temperature could be a factor limiting horizontal transfer *in situ*. Smit and van Elsas (1990) reported that temperature influenced conjugation frequencies when mating studies were carried out with *E. coli*, since lower temperature resulted in a decreased number of transconjugants. Plating at 4°C reduced the number of transconjugants, compared with plating at 20°C and 27°C. They suggested that a lowered metabolic activity of bacteria at lower temperatures might explain the lowered frequency of bacterial mating. Temperature affects microbial activity and the optimum temperature promotes the highest growth and reproduction rate. Generally temperatures near the growth optimum would be expected to promote the maximum conjugal transfer. Trevors and Oddie (1986) showed that in sterile soil and water, plasmid transfer between *E. coli* occurred at temperatures as low as 5°C, but only at very low frequencies and this was attributed either to the loss of donor pili or to the inability of the donor pili to attach to recipient cells at low temperature.

Temperature fluctuations occur in most habitats and most microorganisms have the ability to tolerate some temperature fluctuations. The temperature tolerance of an organism will determine its survivability at temperature extremes but this would not mean that high conjugal transfer could take place over the entire range. Altherr and Kasweck (1982), based on *in situ* studies on the transfer of antibiotic resistant plasmids to *E. coli* K12, reported that transfer was optimal at 25°C and frequencies declined rapidly when the temperature was either raised to 35°C or lowered to 15°C. It can be hypothesised that sufficient population densities if maintained at low temperature, could allow low levels of plasmid exchange and this would lead to metabolic diversity in the population.

Naik et al. (1994) showed that plasmids RP4 and R57.b transferred at high frequencies (10^{-2} transconjugants/recipient) at 30°C, but at low frequencies at 20°C in a number of sterile soils. Most of the studies on temperature-related plasmid transfer used *E. coli* strains, but indigenous soil strains are likely to produce higher transfer frequencies than *E. coli* at more environmentally relevant soil temperatures (Hill and Top, 1998; Fry and Day 1990). Bale et al. (1988) reported that a linear relationship existed between $\log_{10}$ transfer frequency and river temperature (6 to 21°C), with a 2.6°C change in temperature giving a 10-fold change in transfer frequency of the plasmid pQM1.

Lafuente et al. (1996), based on their studies using *E. coli* and *R. meliloti*, reported that *E. coli* with a growth optimum of 37°C would be inhibited in conjugal transfer at 30° to 44°C. This might mean that conjugal transfer is not just dependent on the temperature optimum for the organisms. In our experiment, the maximum number of transconjugants was detected at the temperature range preferred by the donor and recipient (donor and recipient cell densities ~10^9 cfu/g sand between 20° to 25°C). Temperatures higher or lower than the optimal temperature for the organism would also limit conjugal transfer due to reduced donor and recipient cell densities. Temperature also interacts with other environmental parameters and it is necessary to look at the influence of temperature in combination with all other factors. Seeding with a suitable degrader and subsequent transfer of its degradative plasmid might be an effective strategy for bioremediation in sub-tropical climates compared to temperate climes because temperate climates are sub-optimal for high growth of donor and recipients.

3.9.6. Competition experiments with multiple recipients

In nature one to one interactions between microorganisms might not exist. Organisms can potentially act in synergism or compete with each other. In natural systems, biological factors also influence donors, recipients or transconjugants.

In our experiment, transfer of the NAH7 plasmid from *P. putida* PpG7 to *P. putida* KT2440Rif^r, or to kanamycin resistant strains of *P. alcaligenes* DM201 and *P. aeruginosa* AK1414 was studied in pairs, and in combinations to determine the effect of competition in a simple system with naphthalene crystals as nutritional selection and therefore these strains could serve as nutritional or niche competitors without functioning as competitive plasmid-free recipients. The results demonstrated that NAH7 transfer to DM201 and AK1414 remained undetectable even in a one to one mating in sand microcosms (Table 3.4). There was no significant difference in transconjugant numbers between a one to one mating involving PpG7 and KT2440Rif^r and a three way mating involving PpG7, KT2440Rif^r and AK1414. It could be seen in the three way mating involving PpG7, DM201 and KT2440Rif^r, a reduction in the transconjugant numbers and

Table 3.4. Competition experiment with multiple recipients to determine conjugal transfer of NAH7. Values are the mean (± standard deviation) of results from two experiments each plated in triplicate.

Donors and recipients	**Number of transconjugants recovered (cfu/g sand wet weight basis)**
P. putida PpG7 (NAH7) x *P. putida* KT2440Rif^r	$8.1 \pm 0.8 \times 10^{3}$ ^{c}
P. putida PpG7 (NAH7) x *P. alcaligenes* DM201	0 ^{a}
P. putida PpG7 (NAH7) x *P. aeruginosa* AK1414	0 ^{a}
P. putida PpG7 (NAH7) x *P. putida* KT2440Rif^r x *P. alcaligenes* DM201	$7.1 \pm 0.9 \times 10^{2}$ ^{b}
P. putida PpG7 (NAH7) x *P. putida* KT2440Rif^r x *P. aeruginosa* AK1414	$5.0 \pm 1.0 \times 10^{3}$ ^{c}

$^{a\,b\,c}$ Values with the same superscripts are not significantly different as determined by Tukey's HSD test of significance at $P<0.05$

this could be due to the niche competition exerted by the presence of another organism i.e. DM201.

These differences might be due to the different growth rates of the recipients involved in the three way matings. De Rore et al. (1994) based on their work on the transfer of metal resistance plasmid pDN705, reported that high numbers of recipients inhibited extended growth of transconjugants in sterile unpolluted soil and this intraspecific competition was overcome in the presence of heavy metals i.e. in the presence of selection. In non-sterile soils, the effect of heavy metals was not always evident and seemed to berelated to the severity of the selective pressure and inversely to the overall biological competition.

P. alcaligenes DM201 and *P. aeruginosa* AK1414 are apparently not effective recipients. So the only effects they might have are not at the conjugal level per se but perhaps at the metabolic or niche levels by competing for nutrients, producing inhibiting compounds such as siderophores, antibiotics, etc., or colonizing surfaces (occupying niche). This study should be extended to encompass different recipients and different conditions so that the real effect of competition on transfer of NAH7 plasmid can be evaluated.

3.10. Soil microcosm experiments

Sterilized agricultural soil collected from Ellerslie Research Station (University of Alberta) was used as the substrate in the microcosm. The soil experiments were performed at 26°C as described previously (see Section 2.10) with a starting inoculum density of 10^8 cfu/g of soil and a 1:5 donor to recipient cell ratio. Conjugal transfer of the

TOL and NAH7 plasmids were estimated in microcosms with and without nutritional selection.

Both TOL and NAH7 plasmids transferred at low frequencies in sterile soil (Table 3.5) after 24 h of mating (detection limit ca. 30 x 10^1 transconjugants/g of soil). Transconjugant numbers increased in samples after 48 h of mating, which could be attributed to waves of horizontal transfer followed by possible vertical transfer. The selection sources i.e. *m*-toluate and naphthalene added to the soil did not enhance the conjugal transfer of the plasmids.

There are several possible reasons for the low transfer frequencies of TOL and NAH7 observed in soil microcosms. Autoclaving the soil would have resulted in degradation of soil ingredients and also released some soil nutrients and the donor and recipient cells might have proliferated using the readily available carbon in the soil. There would have been no necessity for gene exchange to countervail the selection pressure applied by the presence of *m*-toluate and naphthalene. Selection pressure did not have an effect in these soil microcosms although previous results of the transfer experiments in sterile sand clearly indicated a positive effect due to selection for conjugal transfer of TOL and NAH7 plasmid. Donor and recipient cells survived well in the soil (~10^8 /g of soil).

Klingmuller (1991) reported that addition of sucrose or LB to non-sterilized soil stimulated propagation and elicited plasmid transfer, and van Elsas et al. (1988) observed formation of transconjugants in non-sterile soil only after the addition of a carbon source. Gene transfer frequency was tenfold higher in nutrient-amended soils than in unamended soils (Naik et al., 1994). In addition, the better growth conditions provided by a sandy

Table.3.5. Conjugal transfer of TOL and NAH7 in Ellerslie soil with and without selection. Values are the means of experiments carried out in triplicate (± 1 standard deviation).

	Number of transconjugants (cfu/g of soil wet weight)	
	24 hours	**48 hours**
TOL plasmid		
With selection	<1 x 10^{1}[a]	1.7 ± 0.7 x 10^{3}
Without selection	<3 x 10^{1}[a]	1.1 ± 0.9 x 10^{3}
NAH7 plasmid		
With selection	<3 x 10^{1}[a]	2.1 ± 0.9 x 10^{3}
Without selection	<3 x 10^{1}[a]	1.9 ± 0.8 x 10^{3}

[a] below detection limit

loam soil microcosm supported the maximum conjugation frequencies compared with other types of soil.

It might not be high cell densities alone that promote conjugal plasmid transfer due to cell to cell contact. Strong aggregation of cells to soil particles and non-uniform distribution of donors and recipients might have caused low conjugal transfer of TOL and NAH7 in this study. Another possible factor could be the presence of toxic elements or inhibitory substances in the soil. Autoclaving the soil might also have caused a lot of changes to soil chemistry, especially polymers and phenolics and this might interfere with plasmid transfer.

High clay and silt particle contents in the soil can enhance cell to cell contact soil classification is based on clay, sand or loam content. Some soils might also contain nutrients that are not available to microorganisms. Microbial growth is also restricted to selected favorable surface sites and soil texture is used as an index of available surface area. Soil texture is a measure of the relative concentrations of particles in specific size ranges. Smaller particles have greater surface areas relative to their volumes for microbial colonization (Atlas and Bartha, 1981). If that were the only factor influencing cell to cell contact, then maximum microbial growth and conjugation would have been seen in an agricultural soil but there are a number of other factors that impede growth and cell to cell contact in such soils. Soil from certain ecosystems like an agricultural ecosystem could have high levels of organic matter and this could be toxic to the microorganisms.

Lawlor et al. (1999) observed relatively large differences in the transfer frequencies of metal resistance plasmids between different soils, although the triplicate mating from each replicate showed little variation. They hypothesized that this reflected the inherent heterogeneity of agricultural soil, making comparison of frequencies between different soils difficult. Top et al (1991) demonstrated the positive effect of selective pressure due to the presence of metals in dissemination of heavy metal resistant genes.

Bacteria also do not move appreciably in soil. It is not enough for individual cells to move through macropores, channels or openings made previously by roots, root hairs, earthworms or other soil animals because much of the chemical in soil is at a distance from these channels. The bacteria must be evenly distributed to all sites immediately adjacent to the xenobiotic (Alexander, 1994). In this light, it must be noted that this could also affect cell-cell contact and thereby gene transfer.

Conjugal transfer also depends on the fitness of the isolates. Environmental fitness, which describes the interaction of an organism with its environment, will differ in response to the environment into which it is released (De Leij et al., 1998). Christensen et al. (1998) stated that in more complex environments, newly formed transconjugants might be exposed to a number of environmental signals that could cause repression of pilus synthesis and subsequent suppression of conjugation. They reported that successful establishment of new genetic information was independent of the efficiency with which the donor strain could be established.

Since sterile soil is devoid of competing and synergistic microbes, it does not simulate the normal soil environment. In non-sterile soils, one can also expect plasmid incompatibility to influence gene transfer by excluding new plasmids and limiting gene flow. Biological factors like predators, competition and synergistic effects might also play a role in gene transfer in natural ecosystems.

Trevors et al. (1987) proposed that gene transfer could be studied *in situ* by inoculating the test strains into dialysis sacs (and other environmental chambers) containing soil and these could be sealed and buried in representative locations throughout the test area. In this way, the contained organisms would be subjected to normal environmental fluctuations. In natural environments, at low population densities positive interactions (synergism) would predominate and at high population densities negative interactions are dominant (Atlas and Bartha, 1981). Our study is limiting in taking into consideration only the effect of a few factors and such designs as the above could help us understand conjugal transfer of large catabolic plasmids.

3. 11. Green fluorescent protein (GFP) as a biomarker

Horizontal transfer of catabolic plasmids might be an effective strategy in bioremediation of contaminated sites. It is informative to track the survival of microorganisms after their introduction into a chemically contaminated site to understand the overall success of bioremediation. The sensitive detection of gene transfer from donor strains to indigenous microorganisms in soil by cultural methods requires both efficient marker genes and donor counterselection (Cresswell and Wellington, 1992). The use of a selectable antibiotic resistance marker is disadvantageous in assessing the dynamics of plasmid transfer in soil microbial communities because (a) natural resistance of indigenous recipients can mask the detection of transconjugants (Stotzky, 1989), (b) all the recipient cells must be culturable for such a marker to be used, and (c) it excludes the use of microscopy in monitoring plasmid transfer. Another commonly used method for detecting plasmid transfer in microbial communities is DNA-DNA hybridization (Pickup, 1991) but it cannot provide *in situ* information on conjugal plasmid transfer which occurs in microbial communities. Sensitivity of detection in these hybridizations and mutations or deletions might result in the loss of such markers. The *gfp* gene has been described and used as a reporter gene (Chalfie et al., 1994) and, unlike the *lux* system, GFP detection does not require the addition of a substrate. The fact that the GFP signal can be detected in colonies by simple visual inspection under appropriate wavelengths makes it ideal for high throughput screens (Misteli and Spector, 1997). The main objective of this study was to develop the use of GFP to study transfer of degradative plasmids in microbial communities. This study deals with the use of GFP as a biomarker to detect the transfer of TOL and NAH7 plasmids in colonies arising from culturable transconjugants.

Plasmids and strains used are presented in Tables 2.1 and 2.3. Three strategies were used to achieve insertion of the Tn*5*::*gfp* on to the TOL and NAH7 plasmid: three factor mating, retromobilization, and electroporation.

3.11.1. Constructing a positive control strain expressing GFP

The objective was to obtain a true GFP-expressing clone to use as a positive control for use in determining the sensitivity of GFP detection. The helper *E. coli* strain (RP4) was used to mobilize the suicide vector pSM1373 with the *gfp* gene into the reporter strain, *P. putida* SM1396Nal^r T7 RNA polymerase.

Presumptive transconjugants were selected on PIA with antibiotic counterselection. Donor, recipient and helper controls did not grow on selection plates. The colonies were observed using the low light video imager or luminometer (Figure 3.16), hand-held long wave UV lamp and fluorescence microscopy. The presumptive transconjugants were intensely bright. However, *Pseudomonas* strains which do not express GFP also appear bright due to fluorescein production. This difference in intensity could not be discriminated using the hand held long wave UV lamp but the other two methods, i.e luminometer and fluorescence microscopy, were sensitive probably due to the collection of photons over time, and it was possible to differentiate between the GFP- negative parentals and the GFP- positive transconjugants (Figure 3.16 a, b).

The presumptive transconjugants did not harbor the suicide vector, as revealed by plasmid preparations but the helper plasmid was still present. To overcome this problem of helper transfer, electroporation of the suicide vector into the reporter strain was performed (Section 3.11.4).

The effect of different media on GFP expression from the colonies was also tested. Mineral medium, LB and PIA did not inhibit GFP expression. Addition of Fe_2EDTA as an iron source (5mM) to LB did not help in minimising background fluorescence of *Pseudomonas.* The current study demonstrated that FABC medium (Christensen et al., 1996) might not be suitable for growing colonies for the visualization of green fluorescence (figure not shown) as it did not help differentiate between GFP positive and negative colonies. Mineral medium was also not ideal for obtaining intensely fluorescent colonies that could be detected by the low light video imager but it

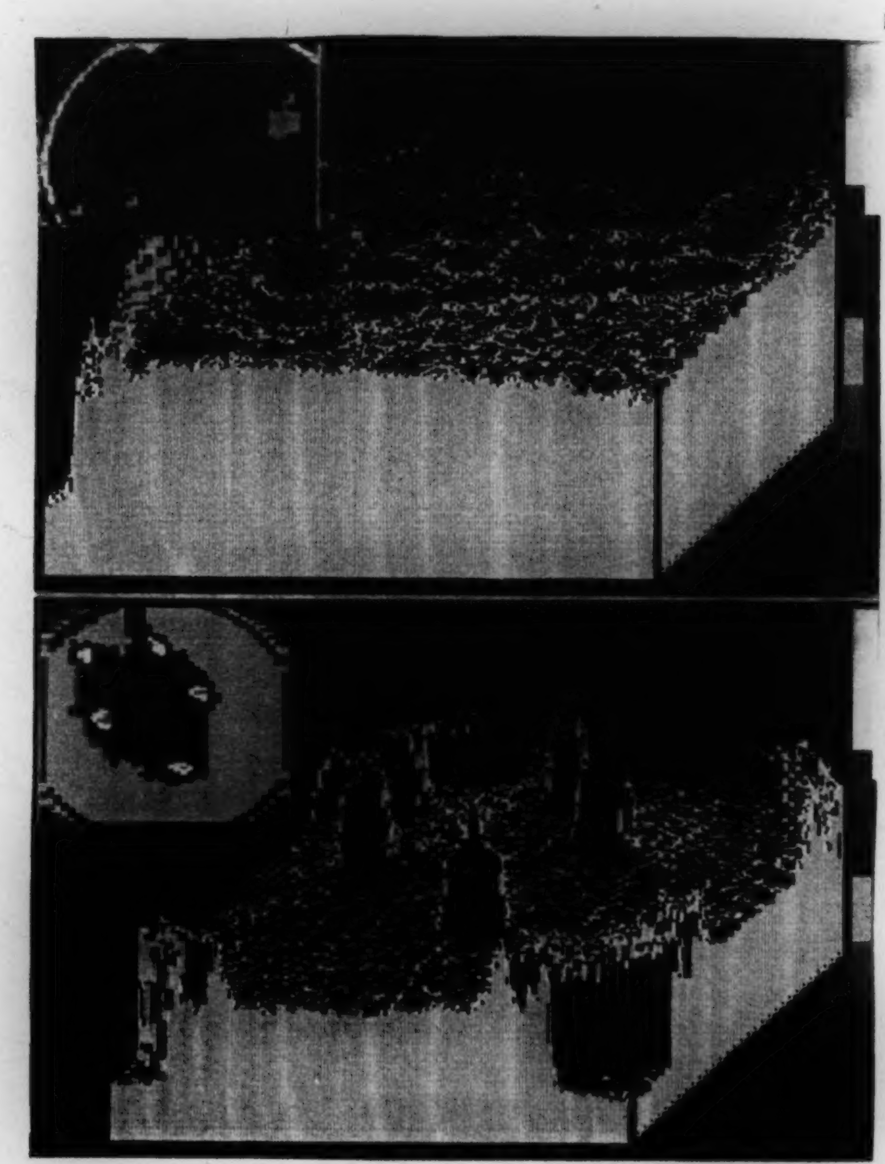

Figure 3.16. Images captured by a low light video imager (Siemens) using a long wave UV light source for GFP excitation. Inset: Agar surface showing fluorescence at an oblique angle, obtained by excitation with UV perpendicular to the agar surface. The "height of colonies" indicates fluorescence intensity. The color bar on the side provides a measure of intensity due to fluorescence. White indicates maximum fluorescence.

Frame a, Parental colonies not expressing GFP
Frame b, Positive control: electroporant colonies expressing GFP

was sensitive enough to differentiate between GFP expressing and non-expressing colonies.

The use of a hand held long wave UV lamp for detecting green fluorescent colonies might result in observing a large number of false positives as *Pseudomonas* produces a high level of background fluorescence when exposed to long wave UV (366 nm). The optimal excitation wavelength for GFP is ~480nm and a long wave UV lamp is not sensitive enough for identifying GFP positive colonies as revealed in this study. It was shown in the current study that the low light video imager is adequate and sensitive enough to detect green fluorescent colonies and the results were comparable to those obtained by using a fluorescence microscope. Sensitivity could be further enhanced by using blue light (470-490 nm) instead of long wave UV to excite GFP expressing cells (Christensen et al., 1996) thereby reducing background fluorescence from pseudomonads. In the current study, single GFP-containing cells were detected by fluorescence microscopy. Such sensitivity in detection could enable the study of time resolved transfer of *gfp*- tagged plasmids.

Christensen et al. (1996) used *gfp* as a marker to study TOL plasmid transfer. Though they detected green fluorescence, a criticism of their work is their usage of the relative term 'intensely bright colonies expressing gfp'. The authors should have screened their green fluorescent colonies and looked for *gfp* tagged TOL plasmids, as they might have been observing the transfer of a *gfp* tagged RP4 helper (resulting from three-factor mating) rather than the *gfp* tagged TOL.

3.11.2. Three factor mating

The helper plasmid RP4 in *E. coli* 6354 was used to mobilize the suicide vector pSM1373 with the *gfp* in Tn*5* into a host harboring the TOL or NAH7 plasmid. Presumptive TOL::*gfp* or NAH7::*gfp* plasmids were subsequently mated into recipient *P. putida* SM1396Nal^r with a chromosomal T7 RNA polymerase gene to promote expression of GFP.

The strategy implemented is described in Section 2.13.1. The transconjugants obtained after the first mating were harvested. The transconjugants could represent the desired transposition event into the catabolic plasmid or could have resulted from transposition of Tn*5*::*gfp* to the helper plasmid for subsequent transfer to the second recipient. In initial experiments, individual transconjugant colonies resulting from transposon mutagenesis were selected and mated with recipient *P. putida* SM1396Nal^r T7 RNA polymerase. Certain predictable events occurred which became clear when the presumptive TOL::*gfp* mutants were screened. Because, these presumptive transconjugants were isolated without *m*-toluate selection, some colonies did not have the TOL plasmid and were *m*-tol$^-$, and only the helper plasmid was seen in the transconjugant, while some contained both the helper and TOL plasmids. Plasmid incompatibility may be responsible for this, as both the TOL and RP4 belong to the same incompatibility group and the RP4 helper may have successfully excluded the TOL plasmid. Presumptives were also selected from PIA containing kanamycin and rifampicin (i.e. double selection) to eliminate the possibility of selecting spontaneous antibiotic resistant mutants of *E. coli* helper strains. Few of the *m*-tol$^+$ presumptive transconjugants from *m*-toluate-containing selection plates harbored two plasmids, of which one was the helper and the other was wild type TOL.

A similar event occurred when the NAH7 plasmid was mutagenized with the Tn*5*::*gfp*. Initially it was presumed that transposon mutagenesis of the degradative plasmid had occurred, and the presumptive transconjugants subsequently mated with *P. putida* SM1396 Nal^r T7 RNA polymerase recipients. The presumptive transconjugants resulting from this second mating were observed using a photo imager and long wave UV observed on mineral medium selection plates containing antibiotics plus *m*-toluate or naphthalene. Green fluorescent transconjugants were observed, presumably with *gfp* inserted on one of the plasmids. However, when plasmid preparations and restriction digestion analysis were performed on these GFP+ colonies, it was apparent that Tn*5*::*gfp* had not integrated into the TOL or NAH7 plasmids (figure not shown). The reason that transconjugants arose on the transconjugant selection plate and not on the donor or recipient control plate might be because of the integration of the Tn*5*::*gfp* Km cassette (5 kb; Figure 3.17 b) into the recipient chromosome instead of the degradative plasmid. Another plausible explanation could be that the *gfp* could have integrated on the helper plasmid and the helper plasmid::*gfp* construct might be responsible for the green fluorescent colonies. In the secondary matings, results were complicated by the fact that donors developed spontaneous nalidixic acid resistance at a high frequency and so nalidixic acid did not prove to be effective in selective for transconjugants only. Nevertheless, it could be that Tn*5* mutagenesis of TOL or NAH7 plasmid itself by three factor mating would not have worked. This strategy suffered from plasmid incompatibility problems resulting from the use of an IncP helper plasmid and, also from low probability of integration of the Tn*5*::*gfp* into the degradative plasmids. Pooling of transconjugants resulting after transposon mutagenesis and using them as donors also yielded similar results i.e. green fluorescent colonies were observed but no appropriate sized plasmids were observed in the transconjugants. Therefore, alternate methods were used to integrate Tn*5*::*gfp* into TOL and NAH7.

3.11.3. Retromobilization

This strategy is based on the assumption that the probability of detecting transposon mutagenesis of TOL and NAH7 would be improved by first carrying out a retromobilization of the suicide vector with *gfp* by the helper plasmid, then transferring the helper and pSM1373::*gfp* into a recipient harboring TOL or NAH7.

The strategy implemented is described in Section 2.13; Figure 2.2. The main problem encountered in the above mating was the spontaneous generation of ampicillin resistant donors during the initial retromobilization of the suicide vector, which made counterselection difficult. Presumptive transconjugants harboring both the helper and the suicide vector were observed, leading to the possibility that unidirectional transfer of the helper plasmid into the donor rather than a bidirectional retromobilization event had occurred. When the ampicillin concentration in selective plates was increased two-fold (to 50 µg/ml), it reduced the frequency of spontaneous ampicillin resistant donor mutants, though not completely. The few presumptive transconjugants which arose were harvested and pooled, grown overnight in LB broth with antibiotics, and mated with *P. putida* KTT1Y(TOL). On selection plates, 60-80 presumptive transconjugants arose on the 10^{-2} dilution plate. These transconjugants were then mated with *P. putida* SM1396Nal^r T7 RNA polymerase, the reporter strain.

Some of the transconjugants (~50%) observed exhibited green fluorescence. This method had the same inherent problems as the three factor mating discussed in Section 3.9.2. i.e. chromosomal integration, or helper mutagenesis with transfer. Plasmid preparation and restriction analysis performed on some of the transconjugants did not reveal Tn*5*::*gfp* integration into TOL or NAH7.

3.11.4. Electroporation

This strategy was adopted to eliminate the problems experienced in the previous two strategies by directly introducing pSM1373 into hosts containing TOL or NAH7 to accomplish transposon mutagenesis. The mutagenized plasmids subsequently were transferred conjugally into the expression host *P. putida* SM1396Nal^r T7 RNA polymerase.

This strategy was the most effective in obtaining Tn*5*::*gfp* insertions, as the mutagenized TOL and NAH7 plasmids were retained in the transformants and ~200 colonies were obtained after the electroporation procedure. GFP-expressing colonies were detected using the low light video imager; the type of medium influenced the degree of fluorescence, with PIA and LB being the best. *Eco*RI restriction digestion of the plasmids obtained from GFP-expressing colonies revealed changes in digestion profiles due to appropriately sized insertions (Figure 3.17 a; discussed in Section 3.11.5). One of the advantages to this method was that the ambiguity due to the presence of a helper plasmid was avoided. This experiment also demonstrated the possibility of transforming an electrocompetent recipient with the large TOL (115 kb) and the NAH7 (83 kb) plasmids.

3.11.5. Evidence that *gfp* is integrated in the TOL and the NAH7 plasmids

The objective of this experiment was to demonstrate that the mutagenized TOL and the NAH7 plasmids generated as described above carry the *gfp* cassette. The TOL::*gfp* and NAH7::*gfp* plasmids were conjugally transferred into a recipient lacking the T7RNA polymerase gene (to show that it is non-GFP expressing, but *m*-tol$^+$ or nah$^+$) and then moved back into a recipient with the T7 RNA polymerase gene (where GFP will be expressed).

Plasmids TOL::Tn*5gfp* and NAH7::Tn*5gfp* were isolated from GFP-expressing *P. putida* SM1396Nal^r and transferred by electroporation into *P. putida* KT2440Sm^r (no T7 RNA polymerase). Plasmids were isolated from the electroporants and subsequently introduced into *P. putida* SM1396Nal^r T7RNA polymerase gene.

Transformants expressed GFP and were *m*-tol$^+$ and nah$^+$, as expected (Figure 3.18; 3.19). It can be seen in Figure 3.18. a. that there were intensely bright and less bright colonies, though both of them do not resemble the fluorescence from the parentals. This difference in brightness could be due to copy number or maturation of the GFP protein. Plasmids isolated from selected transformants harbored plasmids of a higher molecular weight than wild type TOL or NAH7 plasmids in the transformants. Restriction digestion profiles indicated that the Tn*5*::*gfp* had integrated on the degradative plasmids (Figure 3.17 a), as depicted by a change in the banding pattern. It was not possible to determine the molecular weight of the TOL::*gfp* or NAH7::*gfp*

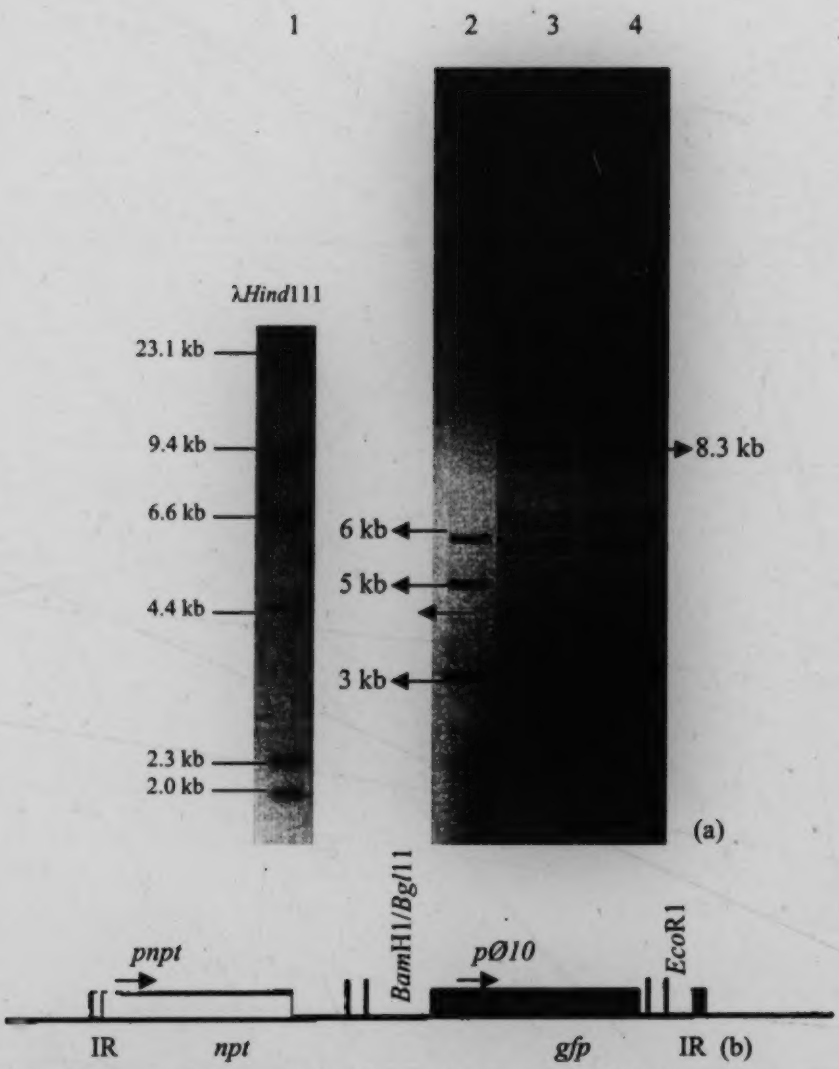
1
2
3
4
λ*Hind*111
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
6 kb
5 kb
3 kb
8.3 kb
(a)
*Bam*H1/*Bgl*11
pnpt
p010
*Eco*R1
IR
npt
gfp
IR
(b)

Figure 3.17. a. Agarose gel electrophoresis after Tn*5*::*gfp* integration into TOL; *Eco*R1 digestions.

Lanes: 1, λ*Hind*111; 2, pSM1373; 3, TOL; 4,TOL::*gfp*

Figure 3.17. b. Representation of transposon vector pSM1373 with *gfp*-containing fragment (Christensen et al. 1996). IR-Inverted repeats; *pnpt*, *p010*-specific promoters;*npt*-neomycin phospho transferase; *gfp*-green fluorescent protein

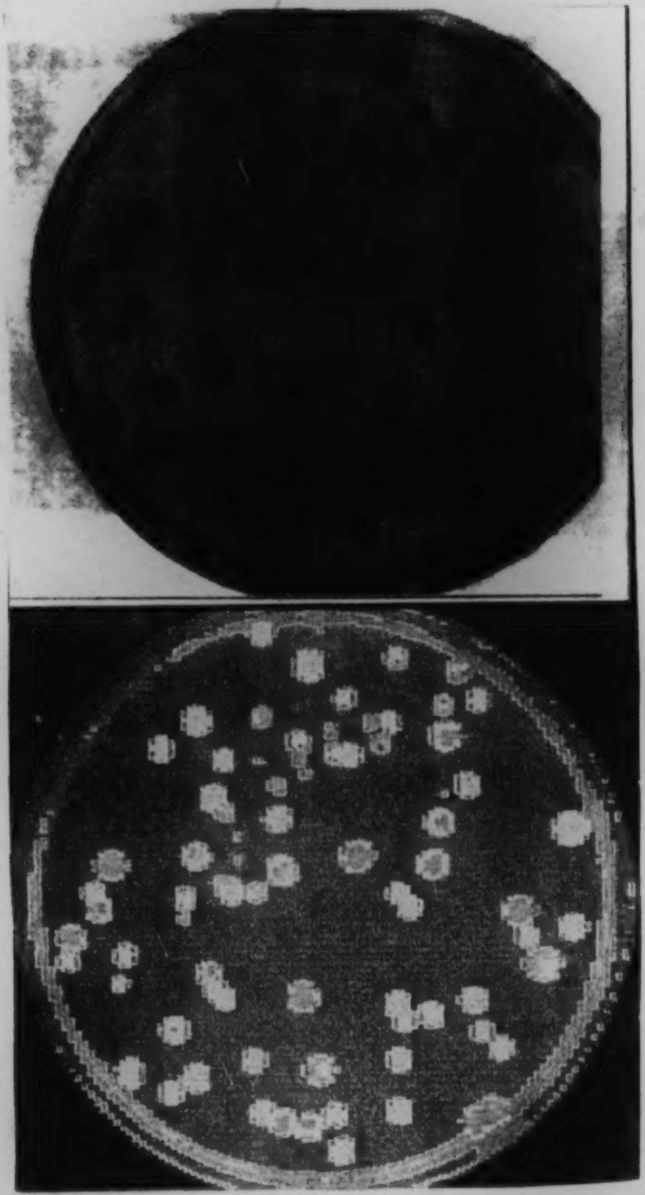

Figure 3.18 Presumptive TOL::Tn*5-gfp* colonies of *P. putida* SM1396Nal^{r} obtained by electroporation. Images were captured by using a low light video imager (Siemens) using a long wave UV light source for GFP excitation. Agar surface showing GFP expressing colonies.

Frame a, Black and white image showing bright GFP expressing colonies on an agar plate.

Frame b, Color image of the same agar plate as seen in Frame a. The bright green colonies express GFP.

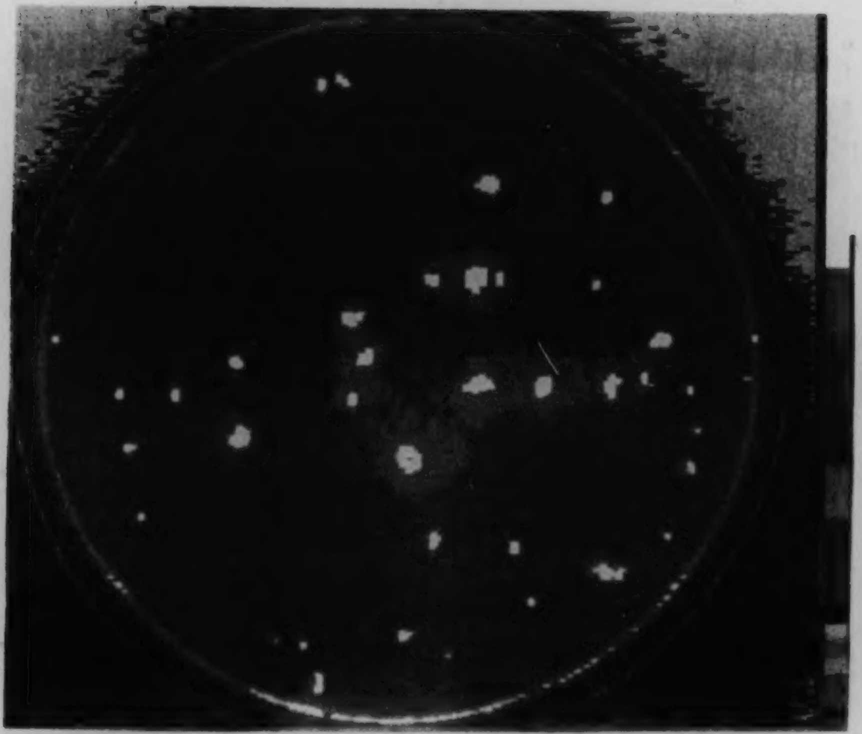

Figure 3.19. Low light image of mature GFP-expressing *P. putida* SM1396Nal^r harboring NAH7::Tn*5*-*gfp*

Images were captured by using a low light video imager (Siemens) using a long wave UV light source for GFP excitation. The color bar on the side provides a measure of intensity due to fluorescence. White indicates maximum fluorescence.

plasmids owing to poor resolution of the lower molecular weight bands and also comigrating doublets. In the case of TOL::*gfp*, there seemed to be an extra ~8.3kb *Eco*RI fragments (Figure 3.17 a) and NAH7::*gfp* was characterized by two ~14 kb and one ~10 kb *Eco*RI fragments (figure not shown). However, both NAH7::*gfp* and TOL::*gfp* could still grow on naphthalene and *m*-toluate. The *gfp* containing fragment in the transposon vector pSM1373 is shown in Figure 3.17 b.

Some of the TOL::*gfp* transformants formed a brown pigment on the *m*-toluate plates. These transformants were also not fluorescent i.e. negative. Plasmid preparations from these transformants revealed a deletion event in the TOL plasmid that differed from the previous ΔTOL (Section 3.3). A part of the *meta* pathway catabolic region could have been deleted from the plasmid resulting in the formation of a catechol intermediate producing the brown polymerization product melanin (for pathway see Figure 1.2). Transposon insertion and subsequent excision would have removed a part of the degradative pathway (only the 18 kb *Eco*RI fragment is missing from the TOL plasmid; figure not shown).

Although Christensen et al. (1996) used *gfp* as a marker for large plasmids and observed fluorescent cells, the authors did not conclusively demonstrate that the Tn*5*-*gfp* gene was indeed integrated on the TOL plasmid. The current study has taken this extra step to demonstrate tagging of the catabolic plasmids with the reporter gene cassette.

Although GFP provides visual confirmation of plasmid transfer, it does not solve the issue of simplifying the procedure of detecting plasmid transfer in a microbial community, especially when dealing with *Pseudomonas* which produces background fluorescence. Newer versions of highly fluorescent mutant GFP or differently colored

GFP might provide a solution, and with refinement it might be a useful tool in detecting gene transfer. Microscopy can also be used for the detection of GFP expressing cells, which would help overcome the problems arising from "viable but non-culturable cells". GFP can be used to trace the movement of a plasmid of interest within a community during plasmid transfer, vertical transfer or cell migration. Better mutants of GFP might be more advantageous than the use of marking systems such as antibiotic resistance and reporter gene systems such as *lacZY-luxAB*. For analysis of heterogenous and complex populations like surface-bound microbial communities there is a need for gene expression reporters that 1. allow for detection at the single-cell level and 2. circumvent the problem of introduction and distribution of chemical substrates for the enzymatic reporter assays. GFP provides an alternative as it fluoresces green and requires the presence of only oxygen to maturate (Chalfie et al., 1994; Andersen et al., 1998). There is also the possibility of using a fluorimeter to quantitate fluorescence that might be useful when dealing with green fluorescent colonies, resulting from plasmid transfer, in biofilms.

Chapter 4

Conclusions

This work is based on the conjugal transfer of the two catabolic plasmids TOL and NAH7. Horizontal transfer by conjugation could be the major contributor to the evolution of new bacterial traits. Microcosms were used to study the effect of different abiotic factors on the conjugal transfer of these large plasmids. Most of the work was performed with *P. putida* mt-2 (TOL) and *P. putida* PpG7 (NAH7) as the plasmid donors and *P. putida* KT2440 as the plasmid recipient.

This study demonstrated interspecific transfer of TOL and NAH7 and expression of the plasmid-encoded catabolic phenotypes in the recipient strain. *P. putida* KT2440 was the most favorable recipient of several strains tested, probably owing to its res⁻mod⁻ property. Transfer frequencies (ca. 10^{-7} transconjugants/recipient) were almost identical when mating was performed in broth or on the surface of agar plates. When TOL transferred to the new recipient, deletion events were commonly observed, resulting in a ΔTOL plasmid, that was 86 kb in size and characterized by the loss of 18 kb, 8 kb and 2.5 kb *Eco*RI fragments compared to the wild type TOL. It was interesting to note that, though the 18 kb *Eco*RI fragment corresponding to *xylAB* gene which encodes xylene oxygenase and benzyl alcohol dehydrogenase, and the 8 kb *Eco*RI fragment corresponding to the *xylS* and *xylR* regulators were absent, the deletion mutants could grow on *m*-toluate as well as xylene. However, it also seems improbable that three different deletion events could occur in the TOL plasmid rather than deletions of contiguous areas. Alternatively, it can be proposed that *Eco*R1 fragments A, P and E corresponding to *xylAB*, and *xylDLEGFJ* could have been lost. But this might not account for the fact that *P. putida* harboring ΔTOL could still grow on the provided substrates. Another strong contender could be the loss of *Eco*R1 fragments A, Q and I. This would also not reconcile with the lowered transfer function of the ΔTOL plasmid In contrast, spontaneous deletions of TOL were not observed in this study even when the original host was maintained on non-selective carbon sources. The ΔTOL plasmids were impaired only in their transfer functions, probably due to the loss of the 2.5 kb *Eco*RI fragment M that corresponds to the region responsible for plasmid transfer and replication functions.

Transfer of NAH7 into *P. putida* KT2440 resulted in apparently stable insertions in the NAH7 plasmid. The resulting +NAH7 plasmid was ~93 kb in size, having acquired an additional 10 kb *Eco*RI fragment. There is a possibility that +NAH7 is larger than ~93 kb but this might be detected only if the occurrence of doublet fragments is resolved. Another insertion NAH7 plasmid of higher molecular weight (~105 kb) was also detected but this plasmid was unstable and transitory. The ~10 kb insertion hybridized strongly to the ~25 kb and 5.9 kb *Eco*RI fragments A and C that correspond to the *nah* operons on the wild type NAH7 plasmid and weakly hybridized to a 7 kb and 2.5 kb *Eco*R1 fragment. The insertion event might contain parts of the *nah* operon. The insertion event did not affect the transfer of the plasmid, but on subsequent transfer the plasmids segregated, resulting in strains harboring either the +NAH7 or NAH7 wild type. These rearrangements, i.e. duplications and deletions might be due to transposition and recombination triggered by transfer to the new host.

Initial parental cell densities influenced the absolute number of transconjugants observed by filter mating and ~10^5 parental cells/mating might represent the lower limits for detectable conjugal transfer on filters. It was also established that presumptive transconjugants resulted from true mating and were not an artefact of subsequent mating on selection plates. When conjugal transfer kinetics of NAH7 were studied, it was observed that transconjugant numbers increased to 10^3 after 800 min (approx. 14 h) of mating and stabilized till 2000 min (approx. 35 h) of mating. After that, transconjugants increased to a cell density of ~10^5 cfu/mating spot at the end of 2800 min of mating. With the TOL plasmid transfer, transconjugants could be detected after 400 min (approx. 7 h) of mating and increased till 1000 min of mating (~10^4 transconjugants). Between 26h and 36 h of mating, the transconjugant density increased 10 fold and this was maintained for the duration of the experiment. The initial increase in transconjugants could be attributed to horizontal transfer and subsequent increase could be due to waves of horizontal transfer (i.e primary and secondary mating events). A six-fold higher conjugal transfer frequency was observed both with a 1:5 and a 1:10 D:R ratio compared with a 1:1 ratio, and this could be due to both horizontal and vertical transfer.

In sand microcosm matings, nutritional selection influenced conjugal transfer frequencies of TOL and NAH7 significantly when compared to matings in the absence of selection pressure. Higher conjugation frequencies were detected only when nutritional selection was provided, which could indicate that gene transfer is required to counter the adverse effects that arise in the environment. Though water facilitates movement of the donors and recipients, moisture contents above 23% did not yield higher transconjugant numbers. A compacted microcosm positively influenced conjugal transfer frequencies compared to a disturbed microcosm, possibly owing to the spatial distribution of donors and recipients. Transconjugant numbers resulting from NAH7 transfer were also influenced by pH of the microcosm. Transconjugant numbers increased in the range of pH 6.6-7.2, whereas transconjugants were not detected at pH 5.8. The pH might affect on plasmid transfer by reducing donor and recipient viabilities such that cell to cell contact is limited. Temperatures higher or lower than the growth optimum modify the physiological state of the bacteria (donor and recipient used) and thereby plasmid transfer might be affected. Optimum transfer of NAH7 was observed at 25°C but transconjugants were still detected at 10°C, albeit at lower frequencies. Temperature also influenced TOL plasmid transfer with the optimum transfer being detected at 30°C. Introduction of a niche or nutritional competitor was performed by introducing three organisms into the microcosm, i.e. *P. putida* strains PpG7 (NAH7), KT2440Rif^r and either *P. alcaligenes* DM201 or *P. aeruginosa* AK1414. *P. alcaligenes* DM201 and *P. aeruginosa* AK1414 are not effective recipients, yet the introduction of DM201 resulted in a decrease in the frequency of NAH7 transfer to KT2440Rif^r. This could be due to competitive effects at the metabolic or niche levels.

In soil microcosms, TOL and NAH7 transfer was undetectable after 24 h of mating, but transconjugant numbers increased after 48 h which could be attributed to limited primary horizontal transfer followed by secondary mating events and possibly vertical transfer.

Green fluorescent protein tagging of TOL and NAH7 could prove to be useful in detecting transfer of these plasmids. Of the different techniques used to mutagenize the TOL and NAH7 plasmid with Tn*5*::*gfp*, electroporation proved to be the most effective. GFP-expressing TOL and NAH7 plasmids could be detected with sensitivity using a low light video imager under long wave UV excitation or a fluorescence microscope.

PAH-degrading bacteria were isolated from contaminated soil samples but the degradative functions apparently were not plasmid-borne, though the environmental isolates harbored multiple plasmids.

This study showed the importance of several abiotic factors on horizontal transfer of TOL and NAH7 plasmids and that gene loss, duplications and deletions might be important processes in evolution.

Future Directions

It would have been interesting to observe mating pair formation microscopically on solid and liquid strata to find out if there were any differences in pili formation especially with regard to TOL plasmid transfer. Electron microscopy studies could also be performed to determine the type of pilus encoded.

The deletion TOL mutants observed in this study did not show any loss of catabolic function. It would be interesting to see if the deleted region in the TOL plasmid in this experiment is due to the presence of these genes on the chromosome of the new host by probing for the presence of the deleted regions. It would be interesting to see if the strains harboring the ΔTOL plasmid is repressed in pilus synthesis. Further experiments should be carried out with TOL transfer into recipients other than *P. putida* KT2440 to determine if deletion events are due to the recipient. Further, it should also be determined if other species carrying the wild type TOL plasmid can transfer the plasmid as efficiently as the original donor. The 2.5 kb deleted region can also be sequenced to determine if it is responsible for transfer functions.

It would be interesting to study the stability of these insertions in NAH7 in a recombination-proficient strain with and without selection pressure. Studies can also be carried out to determine if such insertions are common/frequent when the NAH7 plasmid transfers to other hosts. The necessity for a good physical map for the NAH7 plasmid is also obvious.

Further work could be done using other suitable recipient strains and *gfp*-tagged plasmids to estimate conjugal transfer kinetics of these large plasmids. GFP could enable detection of plasmid transfer microscopically by a time resolved experiment. The results could also be compared with a time course experiment using sand microcosms, although this could be technically difficult due to low expected transconjugant numbers and poor sensitivity of detection in the sand microcosms.

It might be interesting to test the positive effects of selection pressure on NAH7 plasmid transfer using different donors and recipients and also on transfer of other catabolic plasmids eg. "natural" indigenous plasmids.

This experiment could be expanded to encompass greater ranges of pH. Other recipients which grow well at lower pHs should also be used to determine if the low transfer frequencies observed at low pH is not just a function of low cell densities seen with the recipient used.

Similar experiments should be carried out in other types of soil before drawing generalizations about the influence of soil type on conjugal transfer of catabolic plasmids. As a further extension of this research, conjugal transfer should also be estimated in non-sterile soil with and without carbon source. Although study of gene transfer in soil environment is limited by the complexity of the medium (Trevors et al., 1987), the technical difficulty of tracking the plasmid could be solved using GFP-tagged plasmids and fluorimetry for detection.

We could also have used PCR or a ^{32}P-labelled Tn*5* DNA probe to show *gfp* gene integration into the degradative plasmid. In the current study, it was also possible to detect transconjugants resulting from the transfer of TOL::*gfp* and NAH7::*gfp* in sand microcosms.

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Appendix A

A.1. Environmental work

Most work on conjugal transfer has been carried out using antibiotic resistance or degradative plasmids which have been subcultured in the lab for a long period, so they might be inappropriate for drawing conclusions about conjugal transfer in nature. It would be interesting to isolate degradative plasmids from environmental isolates that exhibit multisubstrate degradative ability, and study their conjugal transfer under various conditions.

A.2. Isolation and Enumeration of PAH degraders

There are very few naturally sterile environments on earth and microbes occupy diverse environments. Total heterotrophs in the different soil samples tested (Table 2.4) were ~10^7 cfu/g in EDM, DEV and PAA soils and ~10^6 cfu/g in MTL soil. Only ~10^3 cfu/g of each population could degrade phenanthrene or anthracene, the PAHs most commonly used in this study. Fluorene and chrysene degraders were not detected in the soil samples. The degradative strains (Table A. 1), all of which could grow on phenanthrene, were isolated from enrichment cultures. Other PAH degraders and constituents of microbial consortia in operation in the enrichment cultures might account for the total population density. MTL soil was the most promising in terms of diversity and number of PAH degraders.

All the soils used for the study were contaminated with heavy metals. The total mercury in MTL is approximately 3.5 mg/kg dry soil and the lead content is 220 mg/kg,

Table A.1 Environmental isolates degrading PAHs

Organisms and strain designations	**Phenotype[a]**	**Metal resistance[b]**	**Estimated Plasmid sizes (kb)**	**Source and contamination type**
Sphingomonas paucimobilis Mon Y	Ap^r, Sp^r; Phe$^+$	Cu^{2+}	100, 85, 65	Montreal, Que; petroleum
Pseudomonas sp. Mon 6	Cm^r, Ap^r	Hg^{2+}; Cu^{2+}	-[c]	"
P. vesicularis Edm WPH	Ap^r, Sp^r; Phe$^+$, Ant$^+$, Flu$^+$	Hg^{2+}; Cu^{2+}	85, 65, ~55, 38	Edmonton, AB; creosote
P. aeruginosa Edm WC	Cm^r, Sp^r, Ap^r, Tc^r; Phe$^+$	Hg^{2+}; Cu^{2+}	~100	"
P. mesophilia Dev	Cm^r, Phe$^+$	Hg^{2+}	~50, ~38	Devon, AB; petroleum
P. aeruginosa PAA	Cm^r, Sp^r, Ap^r, Tc^r, Sm^r; Phe$^+$	Hg^{2+}; Cu^{2+}	-[c]	Prince Albert, SK; creosote

[a] Phe$^+$- utilization of phenanthrene as C source
Ant$^+$- utilization of anthracene as C source
Flu$^+$- utilization of fluoranthene as C source

[b] Mercury and copper resistance as measured using metal agar plates (Appendix B)

Cm, Ap, Sp, Tc, Sm- Antibiotic resistance exhibited by the organisms

[c] no plasmids detected

approximately 100 times and 14 times higher than typical non-contaminated soils respectively (M. A. Pickard, personal communication). Such high concentrations of heavy metals in soils might select for the presence of heavy metal resistance genes in the indigenous microflora, although genes conferring resistance to Cu and Hg are also present in agricultural soils. Quantitative resistance levels of the isolates to Cu^{2+} and Hg^{2+} is presented in Table A.2.

Spray plates or agarose overlay plates supplying PAHs as sole carbon source are differential as well as selective, because PAH-utilizing colonies will produce surrounding zones of clearing or color, enabling their detection. However, the method has limitations, as it can favour the isolation of fast growing isolates if the plates are incubated for a short (< 1 week) period. It also precludes isolation of organisms unable to grow on the medium used (i.e. mineral salts or Bushnell-Haas media lacking cofactors, vitamins, etc.,). If there are a number of species with similar physiological characters in the enrichment cultures, then the microorganism with the fastest growth rate will dominate. Some microorganisms might not be isolated if they do not grow in pure culture, as they might need specific growth factors produced in mutualistic relationships. Therefore, the isolates described in Table A. 1 may not represent all potential PAH degraders in the soil.

A.3. Characterization of the PAH degraders

Biochemical tests and API 20NE strips revealed that almost all the organisms isolated from the contaminated sites (Table A.2) were pseudomonads as reported by others (Ogunseitan et al., 1987; Sayler et al., 1990).

Table A 2 . Resistance of the environmental isolates to Cu^{2+} and Hg^{2+} provided as copper sulphate and mercuric chloride in agar plates.

COPPER			Growth[a]		
mM	Mon Y	Edm WC	Edm WPH	PAA	Dev
0 (control)	+	+	+	+	+
0.01	+	+	+	+	-
0.05	+	+	+	+	-
0.1	+	+	+	+	-
0.5	+	+	+	+	-
1	+	+	+	+	-
2.5	+	+	+	+	-
5	+/-	+	+	+	-
10	+/-	-	-	-	-
20	+/-	-	-	-	-
30	-	-	-	-	-
40	-	-	-	-	-

MERCURY			Growth[a]		
mM	Mon Y	Edm WC	Edm WPH	PAA	Dev
0	+	+	+	+	+
0.01	+	+	+	+	-
0.05	+	+	+	+	-
0.1	+	+	+	+	-
0.5	-	-	-	-	-

[a] +, good growth; -, no growth; +/- weak growth compared with growth on control plates

Extracted methanol-soluble pigments from MonY showed absorbance spectra comparable to nostoxanthins described previously (Jenkins et al., 1979; Pollock, 1993), with a major peak at 448 nm, a secondary peak at 475 nm and a minor inflection at 430 nm classifying the strain MonY as *Sphingomonas paucimobilis*.

The high incidence of *Pseudomonas* in contaminated sites studied by Clarke (1973) was attributed to the regulation system of *Pseudomonas* having more flexible responses to novel substrates. The catabolic pathways of *Pseudomonas* appeared to be composed of discrete functional units controlled by genetic modules that evolved through genetic transfer and rearrangement. In a study conducted in New Zealand the lower molecular weight aromatics such as phenanthrene and carbazole were degraded by Gram-negative pseudomonads, whereas the higher molecular weight compounds such as fluoranthene and pyrene were degraded by Gram-positive rod-shaped bacteria belonging to a *Mycobacterium* species cluster (Aislabie et al., 1997). Berardesco et al. (1998) identified members of several genera including *Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Flavobacterium*, *Vibrio* and *Mycobacterium* as a community of PAH-degrading bacteria in a single 0.5 g sediment sample. They stated that the high diversity is most likely because phenanthrene degraders were selected from primary spread plates instead of enrichment cultures, which select for only the strains growing most rapidly under laboratory conditions. Soil microbial populations, particularly *Pseudomonas* spp., have evolved considerable nutritional versatility and are capable of degrading a range of complex, naturally occurring aromatic and aliphatic compounds (Gunsalus and Marshall, 1971; Don and Pemberton, 1981). Our results did not isolate any Gram positive PAH degraders from the contaminated sites. This might be either due to the fast growing nature of the pseudomonad-like organisms preventing the detection of Gram positives, or it could be because of a genuine absence of PAH-degrading Gram positives in the enrichment cultures. That is, the enrichment culture procedure might have favored pseudomonads. Another plausible explanation could be that the Gram positives in the soils could possess partial catabolic pathways and thus might thrive only as members of a consortium.

A.4. Endogenous plasmid isolation

Plasmid isolations were carried out with the PAH degraders (see Section 2.10; and Appendix C). Many of the environmental isolates harbored more than one plasmid, verified by two-dimensional gel electrophoresis (Table A.1). Many of the plasmids isolated from the degradative strains were large (Table A.1; Figure A.1) like the typical degradative plasmids.

Plasmids are divided into incompatibility groups based on their replication control or partitioning functions. Compatible plasmids can co-exist in the same cell as independent replicons for many generations without either of them being lost. The multiple plasmids present in the environmental isolates were stably maintained, indicating that they were compatible and probably belonged to different incompatibility groups. The presence of multiple plasmids might represent the process of evolution. The understanding of microbial gene transfer, including how bacteria acquire and disseminate genes, and retain multiple plasmids will provide us information on the role of horizontal transfer in bacterial evolution.

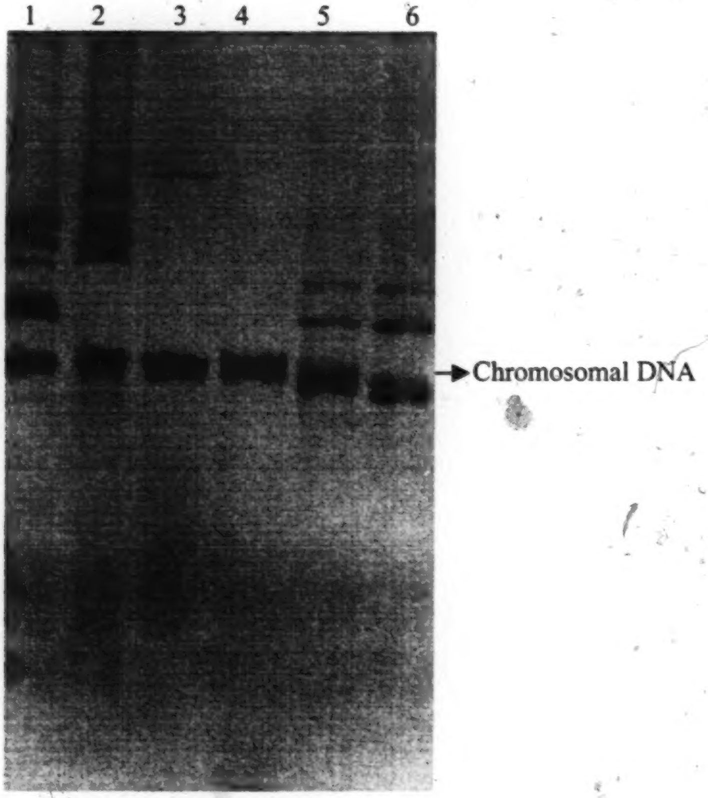

Figure A 1. Plasmids present in environmental isolates from creosote and and petroleum contaminated sites.. For plasmid sizes see Table A.1. Lanes: 1, *P. vesicularis* EdmWPH; 2, *S. paucimobilis* MonY; 3, *P. aeruginosa* EdmWC; 4, *P. aeruginosa* PAA; 5, *P. mesophila* Dev-1; 6, *Pseudomonas* sp.Dev-2

A.5. Conjugation studies and probing for degradative genes

P. putida KT2440Rif^r (*m*-tol$^-$, nah$^-$) was used as a recipient for detecting incoming degradative plasmids. Attempts to transfer the plasmids present in the degradative soil isolates to *P. putida* KT2440Rif^r by standard filter mating did not yield transconjugants on the selection plates. Possibly the plasmids present in *S. paucimobilis* MonY and *P. vesicularis* EdmWPH do not carry the phenanthrene catabolic genes and hence transfer could not be detected on the selection plate; alternatively the plasmids may not be self-transmissible. *P. putida* was chosen as the recipient because most degradative plasmids are found in pseudomonad-like organisms, however *P. putida* KT2440Rif^r might not be a suitable expression host for the catabolic plasmids, or conjugation conditions may have been unsuitable.

The plasmids were not cured even after fifteen transfers in LB medium, indicating that they might be essential for the survival of the host, or that they have efficient partitioning mechanisms. The number of transfers carried out in this experiment may have been too low to cure the organisms of their plasmids, as A. Hohnstock (personal communication, 1998), found that 90 transfers were required to cure a catabolic plasmid from *Pseudomonas* sp.

Southern blots and DNA-DNA hybridizations revealed that the pGJZ1707 probe (Goyal and Zylstra, 1996), bearing genes for phenanthrene dioxygenase and phenanthrene *cis*-dihyrodiol dehydrogenase did not hybridize with plasmid DNA from any of the environmental isolates. This might show that the environmental isolate plasmids do not encode phenanthrene degradative genes or, due to their metabolic diversity arising as a result of evolution, might have genes that bear litlle homology to the probe. For example, three non-homologous families of genes responsible for phenanthrene degradation were found in *Comamonas testosteroni* strains isolated from the same environmental sample, implying the existence of divergent degradative genes (Goyal and Zylstra, 1996). Dahlberg et al. (1997) reported that plasmids from adjacent microhabitats bore little functional resemblance to each other but there were high degrees of DNA-DNA homology between plasmids from different areas.

Plasmids that do not confer a selective advantage are usually lost through competitive exclusion (Slater et al., 1980; Hardman et al., 1986). However, even when selective pressure is absent, a plasmid might be maintained at a low frequency in a population providing a 'community memory' of a prior successful strategy (Boumam et al., 1988; Jain et al., 1987). Cells are able to carry genetic capability as cryptic genes that may be activated under appropriate environmental conditions or merely by chance. The 'metabolic burden' associated with a cell harboring such genetic material, as determined by a decrease in growth rate, is caused by plasmid replication, transcription of mRNA, and foreign protein expression (Terzaghi and O'Hara 1990; Bentley et al., 1990). If nonessential traits are harbored on plasmids within a few species, then the metabolic burden would be shared among members of the community. These plasmids would also confer an adaptive flexibility in terms of substrates to a plasmid carrier population (Rittman et al., 1990; Boyle, 1992).

Degradative plasmids tend to be large in size, and include composites, which can dissociate into separate autonomously replicating plasmids upon transfer into certain strains (Bradley et al., 1986; Chakrabarty et al., 1978). Specific probes such as NAH7 or cloned degradative genes could be useful for assessing naphthalene degrading populations or specific degradative genes in the environment but they probably underestimate the real number and variety of PAH-degradative strains (Foght and Westlake 1991). DNA-DNA hybridization results reported by Berardesco et al. (1998) indicated that naphthalene dioxygenase and the *P. putida* NCIB 9816 phenanthrene- and naphthalene-degradative genes play only a minor role in some envrionments. They also found that a single analysis of a natural community is not sufficient to characterize a degradative community. It might be interesting to study hybridization patterns of the plasmids as well as chromosomal DNA of the environmental isolates from the current study with other known degradative probes.

A.6. Exogenous plasmid isolation

Experiments attempting exogenous plasmid isolation have yielded mixed results. Triparental exogenous isolation is a method which enables selection of plasmids based on conjugative properties as a phenotype (Top et al., 1994). Plasmids able to replicate in a number of Gram negative bacterial species, designated "broad host range plasmids", are very important in horizontal transfer. Host range is also important if plasmids are to spread throughout the members of a natural community. Most exogenously isolated plasmids from water seem to be broad host range (Day and Fry, 1992). *P. putida* KT2440Rif^r strain and *E. coli* JE 2571 (RP1) were used as the recipient and helper stains respectively in the current study.

In the current study on exogenous plasmid isolation, transconjugant selection plates as well as the donor control plates showed growth (10^4–10^6 cfu/mating), whereas the recipients did not grow on the selection plates. The difficulty encountered with this method was the high antibiotic resistant donor background (see Table A.1), which made donor counterselection difficult. It was also difficult to differentiate between the transconjugants (if any) and the soil bacteria from suspension of the donor strains plated onto the transconjugant selection plate. Plasmid preparations on colonies picked from transconjugant selection plates did not reveal plasmids, suggesting that the indigenous organisms might have only chromosomally encoded catabolic and antibiotic resistance genes.

Retromobilization also failed to recruit any non-self transmissible catabolic plasmids. The donor control and the transconjugant selection plates showed growth, leading to the same problems as encountered earlier. In one particular instance, when MTL soil was used as the plasmid donor source, a few colonies (40-50 on a 10^{-3} dilution transconjugant selection plate) which were not morphologically similar to the recipient, were found to harbour a plasmid identical in size to the RP1 helper plasmid. Either the RP1 plasmid used for mobilization moved in the classical forward direction or indigenous soil microbes might be harboring a plasmid similar in size to RP1.

Exogenous isolation was successful when NAH7 or the R3006 plasmids were used as the transmissible plasmids in positive control experiments carried out in sand microcosms. The reasons for failure of this technique with indigenous plasmids could be that the number of conjugative or self-transmissible catabolic plasmid-containing strains or the transfer frequency of these plasmids might be too low to be detected. A lack of expression of catabolic genes in the recipient could also have hindered their detection. Other unknown factors such as soil conditions, donors not being adequately dislodged from the soil, or inappropriate growth conditions might also have hindered this technique.

It could also be due to the absence of catabolic plasmids in the environmental samples taken for this study, as illustrated in the results of the previous experiments.

A reported attempt to exogenously isolate antibiotic resistance plasmids from soil ecosystems was also unsuccessful (van Elsas et al., 1989). Despite the presence in polluted sites of degradative strains harboring catabolic plasmids, these plasmids could not be readily isolated from these ecosystems by exogenous isolation. A narrow transfer, replication or expression range could be the reason for the unsuccessful nature of these experiments (Top, 1993).

The current study suggests that, although degradative strains are present in the environment, the degradative functions might not be plasmid-borne. Environmental strains harbor multiple cryptic plasmids and further work is required to determine the significance of these plasmids in the environmental isolates.

APPENDIX B

ANTIBIOTICS USED

	Stock	Working concentration
Chloramphenicol	30 mg/ml in ethanol	30 μg/ml
Kanamycin	25 mg/ml in ddH_2O	50 μg/ml
Tetracycline	12.5 mg in 1:1 ethanol/water	12.5-15 μg/ml
Ampicillin	25 mg/ml in ddH_2O	35-50 μg/ml
Streptomycin	20 mg/ml in ddH_2O	25 μg/ml
Spectinomycin	40 mg/ml in ddH_2O	100 μg/ml
Rifampicin	25 mg/ml in methanol	150 μg/ml
Nalidixic acid	40 mg/ml in water	100 μg/ml

All the antibiotic stock solutions were filter sterilized and stored at –20°C.

BIOCHEMICAL TESTS

Catalase test

To a couple of drops of 5% hydrogen peroxoide in a test tube, smear the unknown culture. The presence of bubbles indicates a positive reaction.

Oxidase test

To a drop of TMPD (tetramethyl-*p*-phenylenediamine) reagent added to a filter paper, aseptically remove the culture to be tested and mix. A positive reaction gives a purple color within 10 seconds.

Electroporation recovery medium (S. O. C. medium)

Solution I

Tryptone	2 g
Yeast extract	0.5 g
NaCl	0.06 g
HCl	0.02 g

Dissolved in ddH_2O to 90 ml final volume, then autoclaved for 20 min.

Solution II

$MgSO_4 \bullet 7H_2O$	2.46 g
$MgCl_2 \bullet 6H_2O$	2.03 g

Dissolved in ddH_2O to 50 ml final volume, autoclaved for 20 min.

Solution III

Glucose	0.18 g

Dissolved in ddH_2O to 50 ml final volume, autoclaved for 10 min.

Aseptically add 5 ml of both solutions II and III to solution I.

MEDIA

All the media used were prepared by dissolving the constituents in doubly distilled water, sterilizing by autoclave for 20 minutes at 121°C under liquid cycle. If antibiotics were used, they were added to the media only after cooling to 50°C in a water bath. All media used were stored at 4°C in the dark. The components listed below are per litre of distilled water unless otherwise noted.

Bushnell-Haas (Difco manual, 1968)

$MgSO_4 \bullet 7H_2O$	0.2 g
$CaCl_2 \bullet 2H_2O$	0.02 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
NH_4NO_3	1.0 g
$FeCl_3 . 6H_2O$	0.05 g
Bacto agar (Difco)	15 g for solid medium

If the optional C source was water soluble, then it was dissolved directly in the mineral medium or BH medium. If not, a spray plate technique (Kiyohara et al., 1982) was adopted.

FABC medium (Christensen et al., 1996)

$MgCl_2$	1 mM
$CaCl_2$	0.1 mM
FeEDTA	0.01 mM
$(NH_4)2SO_4$	0.15 mM
Na_2HPO_4	0.33 mM
KH_2PO_4	0.2 mM
NaCl	0.5 mM
Sodium citrate	10 mM
Bacto Agar	20 g

Luria Bertani broth (LB)

Bacto-tryptone	10 g
Yeast extract	5.0 g
NaCl	10 g
Bacto-agar	15 g

pH adjusted to 7.5

LB agar

LB broth	1 L
Bacto agar	15 g

Mineral medium

K_2HPO_4	4.4 g
KH_2PO_4	1.7 g
NH_4Cl	2.1 g
100 X salt solution	10 ml
Agar noble (Difco)	15 g for agar plates

100 x salt stock

$MgSO_4 \cdot 7H_2O$	19.5 g
$MnSO_4 \cdot H_2O$	5.0 g

$FeSO_4 \bullet 7H_2O$	5.0 g
$CaCl_2 \bullet 2H_2O$	0.3 g
Ascorbic acid	1.0 g

Filter sterilized and stored at 4°C

10 MM PHOSPHATE BUFFER PREPARATION

100 x phosphate buffer stock

KH_2PO_4 (1M) 20.3 g in 150 ml ddH_2O

K_2HPO_4 (1M) 43.5 g in 250 ml ddH_2O

The mono-potassium phosphate solution was added to the di-potassium phosphate solution until a pH of 7.2 to 7.3 was achieved. This stock was then diluted 1:100 with ddH_2O for a final concentration of 10 mM phosphate buffer, and then autoclaved.

METAL RESISTANCE DETERMINATION (Nieto et al., 1989)

Mercuric chloride ($HgCl_2$, 273 mM) and copper sulphate ($CuSO_4 \bullet 5H_2O$, 600 mM) were dissolved in ddH_2O and used as stock solution to give final metal concentrations ranging from 0 mM to 40 mM.

Mineral medium +

Glucose	2 g
Bacto-agar	15 g

The metal stock solution was added to the sterilized medium at 50°C.

BUFFERED MEDIA FOR PH ANALYSIS

This mixed buffer system was prepared by mixing the appropriate amounts of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4 as indicated below. The final volume was made up by adding LB or mineral medium to bring the volume to 50 ml.

pH	0.2M NaH_2PO_4 (ml)	0.2 M Na_2HPO_4 (ml)
5.8	23.0	2.0
6.0	21.9	3.1
6.2	20.4	4.6
6.4	18.4	6.6
6.6	15.6	9.4
6.8	12.8	12.2
7.0	9.8	15.2
7.2	7.0	18.0
7.4	4.8	20.2
7.6	3.3	21.7
7.8	2.1	22.9
8.0	1.3	23.7

MEDIA FOR PAH DEGRADERS

PAHs cannot be incorporated in agar by dissolving them in water. The methods used are:

Agarose overlay plates (Bogardt and Hemmingsen, 1992)

The plates are made of two layers. Zones of clearing due to PAH-degraders in the upper layer are easier to visualize if the mineral salts agar underlayer is kept as clear as possible by using high-purity agar (Agar Noble, Difco). For the upper layer, 1% low-gel-temperature agarose is prepared using the mineral salts medium; after cooling to 30 to 35°C, 0.2 ml of 8.5 mg of PAH per ml of ethanol is added to 3.5 ml of agarose. They are mixed immediately, and then 0.1 to 0.2 ml of the bacterial dilution is added and mixed. The agarose is then poured over the prepared underlayer and plates are allowed to harden.

Spray plates (Kiyohara et al., 1982)

Mineral agar plates are sprayed with a 1 to 10% solution of PAH in ether or acetone. An atomizer (Jetpak) was used to obtain a fine mist to obtain even coverage. The plates can be sprayed after inoculation of the plates. These plates are differential because colonies because the colonies which can utilize the PAH will produce either zones of clearing or colored zones around them. Overspraying of the plates should be avoided as too much solvent can kill the bacteria or cause excessive colony spreading.

For naphthalene, the air can be saturated with naphthalene vapors by adding crystals to the plate lids.

APPENDIX C

CHEF-PFGE
GENERAL PROTOCOL (Netolitzky, 1995)

Agarose plug sample preparation

Overnight cultures were pelleted by centrifugation and the pellets were resuspended in an equal volume of 0.25 M EDTA (pH 8). Lysozyme was added to a final concentration of 0.5 mg/ml and the suspension was incubated at 37°C for 30 min. During this time a 1% solution of low gelling agarose in 0.25 M EDTA was melted and allowed to cool to 50°C. This was then added to the cell-enzyme suspension to achieve a final agarose concentration of 0.75%, adding additional 0.25 M EDTA if required. The suspension was pipetted into the CHEF chamber comb and allowed to set at 4°C for 20 min.

The agar plugs containing the cell-enzyme suspension were placed into a petri dish with enough LET buffer to cover the plugs and incubated overnight at 37°C. The LET buffer was removed and the plugs washed three times for 15 mineach in 0.05 M EDTA (pH 8). SDS solution was then added to the plugs and this was then allowed to incubate overnight at 50°C. The SDS solution was removed and the plugs are washed for 15 min and again overnight in 0.05 M EDTA (pH 8) at room temperature. A final wash in 0.05 M EDTA (pH 8) was conducted for 15 min at room temperature and the samples could then be stored at 4°C in this buffer until used.

PFGE

The agarose plugs containing the lysed cell samples were cut with a glass coverslip and placed into the wells of a prepared gel up to 90% of the well height. Similarly the desired amount of λ ladder DNA was also cut and placed into the well. The wells were then filled with a molten solution of 1% agarose in 0.5x TBE and allowed to set. The gel was placed in the apparatus and rested on the gel stops. The gel was covered with 1 to 2 mm of 0.5 M TBE. Liquid samples can be added to the gel at this time. A pump was used to circulate and cool the buffer (3 L required) through a cooling chamber and the gel apparatus at a flow rate of one litre per minute, allowing the gel to be run at a temperature of 14°C. The gel should be run for 10 min prior to the pump being turned on, to facilitate entry of the samples into the gel. For this analysis, the gel apparatus was set to run for 24 hours with a ramped pulse of 165 volts increasing over time from 5 to 80 seconds. The gel was stained and visualized for as other gels.

λ DNA ladder (New England Biolabs)

molecular weight marker, 50 µg/ml

50 to 1000kb

supplied in an agarose plug within a syringe dispenser (10 mM Tris-HCl, pH 8, 1 mM EDTA, 50% glycerol), ready to cut and insert into gel wells.

LET buffer

EDTA (pH 8)	0.45 M
Tris (pH 7.5)	0.01M

2-mercaptoethanol	7.5%

SDS solution

Tris (pH 7.5)	0.01 M
EDTA (pH 8)	0.45 M
SDS	1%
Proteinase K	1 mg/ml

REAGENTS FOR MOLECULAR STUDIES

CASSE PLASMID DNA PREPARATION SOLUTIONS (Casse et al., 1979)

Equilibrated phenol

Top quality crystalline phenol was melted at 55°C and mixed with an equal volume 50 mM Tris:20 mM Na_2EDTA:1 M NaCl (pH 8). The phases were then allowed to separate and the organic phase was used immediately.

Lysing solution

4% SDS in 50:20 TE

adjust to pH 12.4 using 10 N NaOH; prepare freshly

Neutralization solution

2 M Tris-Hcl; pH 7

Wash solution

50 mM Tris:20 mM Na_2EDTA; pH 8

MARMUR CHROMOSOMAL DNA PREPARATION SOLUTION (Marmur, 1961)

Saline EDTA

NaCl	0.15 M
Na_2EDTA	1 M

pH 8

MODIFIED KIESER PLASMID DNA PREPARATION SOLUTIONS (Kieser, 1984)

Acid phenol/chloroform

Crystalline phenol	5 g
Chloroform	5 ml
8-hydroxyquinoline	5 mg
ddH_2O	1 ml

Lysis buffer

Sucrose	10.26 g
Tris (1M)	2.5 ml
Na_2EDTA (0.5 M)	5.0 ml

Made up to 100 ml

Lysis solution

NaOH	0.3 N
SDS	2% w/v

GEL ELECTROPHORESIS BUFFERS

50x TAE stock solution

Tris	242 g
Na_2EDTA (0.5M)	100 ml
Glacial acetic acid	57.1 ml
Made up to 1 litre	pH 8

5x TBE stock solution

Tris	54 g
Na_2EDTA (0.05M)	20 ml
Boric acid	27.5 g
Made up to 1 litre	pH 8

0.5 M EDTA (pH 8)

Na_2EDTA• 2H2O	181.6 g
ddH_2O	800 ml

pH adjusted to 8.0 with ~20 g NaOH pellets and sterilized by autoclaving

Lysozyme

Stock 50 mg/ml (in ddH_2O)

Store at –20°C

5x loading dye

50% glycerol

0.1 M Na_2EDTA, pH 8

0.25% bromophenol blue

0.25% orange G (optional RNA marker)

0.25% xylene cyanol (optional high molecular weight DNA marker)

filter sterilize solution

SOUTHERN HYBRIDIZATION

Denhardt's reagent (Sambrook, et al., 1989)

Made up as a 50x stock, filtered and stored at –20°C

Stock is diluted ten-fold into pre-hybridization buffer

50x Denhardt's reagent contains:

Ficoll (Type 400, Pharmacia)	5 g
polyvinylpyrollidone	5 g
bovine serum albumin (Fraction V, Sigma)	5 g

H_2O to 500 ml

Pre-hybridization solution recipe (Amersham)

ddH_2O	15.0 ml
20x SSPE	6.25 ml
50x Denhardt's	2.5 ml
10% (w/v) SDS	1.25 ml

20X SSC

3 M NaCl, 0.3 M trisodium citrate, adjusted to pH 7.0 with 1 M HCl.

20x SSPE (Sambrook et al., 1989)

dissolve 175.3 g of NaCl, 27.6 g of NaH_2PO4. H_2O and 7.4 g of EDTA in 800 ml of ddH_2O

adjust the pH to 7.4 with NaOH (~6.5 ml of a 10N solution)

adjust the volume to 1 litre with water

dispense into aliquots, sterilize by autoclaving